Preclinical evaluation of pharmacological strategies designed to enhance the activity of established and novel anti-cancer drugs.

Synopsis:

Evaluation of pharmacological strategies designed to modulate the Warburg effect, enhance the activity of tyrosine kinase inhibitors and novel analogues of Temozolomide.

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ABSTRACT

Whilst progress has been made in reducing mortality in some cancers, mortality rates remain high in many cancers and there is a need to develop novel therapeutic strategies. In this thesis, various pharmacological strategies designed to enhance the activity of existing therapeutic drugs were evaluated. Cancer cells are dependent upon aerobic glycolysis (the Warburg effect) and glutamine uptake. Using clinically approved tyrosine kinase inhibitors and Bortezomib, significant enhancement of chemosensitivity was observed when used in combination with inhibitors of lactate dehydrogenase (Gossypol) and pyruvate kinase dehydrogenase (Dichloroacetate). In contrast, depletion of glutamine from media had to be extensive in order to induce cell death and cell death only occurred after prolonged exposure to glutamine-deprived conditions. This suggests that glutamine depletion strategies alone are unlikely to be successful but may be useful in combination with other agents targeting glutamine addiction in cancer cells. Finally, Temozolomide (TMZ) is an important drug in the treatment of glioblastomas but its activity is reduced by resistance mechanisms including O^6 methyl guanine methyltransferase (MGMT) and mismatch repair (MMR). This thesis has identified analogues of TMZ (EA02-45, EA02-59, EA02-64 and EA02-65) that are MGMT and MMR independent in terms of inducing cell kill *in vitro*. These compounds are promising leads for future development. In conclusion, this thesis has demonstrated that interfering with the metabolic phenotype of cancer can enhance the activity of existing drugs and identified novel analogues of TMZ that circumvent drug resistance mechanisms that hamper the efficacy of TMZ.

Key words: Warburg effect, glutamine metabolism, tyrosine kinase inhibitors, dichloroacetate, pyruvate dehydrogenase kinase, temozolomide, chemosensitivity

Acknowledgement

I would like to dedicate this thesis to my parents.

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Abbreviations

^{18}FdG/^{18}FDG - ^{18}fluorodeoxyglucose

AFPGC - AFP producing gastric cancer

AGT - O^6-methylguanine O^6-alkylguanine transferase

αKG - α-ketoglutarate

ALL - Acute lymphoblastic leukemia

AOS - Activated oxygen species*

APAF1 - Apoptosis protease activating factor 1

ATCC - American tissue culture collection

ATP - Adenosine triphosphate

BSA - Bovine serum albumin

CT - Computed tomography

DCA - *Dichloroacetate*

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

DTIC - Dacarbazine

ECACC - European Collection of Animal Cell Cultures

ETC - Electron transport chain

FasL - Fas ligand

GDH - Glutamate dehydrogenase

GLS - Glutaminase

GMB - Glioblastoma multiforme

HBSS - Hanks Balanced Salt Solution

HIF - Hypoxia inducible factor

HIF-1 - Hypoxia inducible factor-1

Kv - Potassium voltage

LDH - Lactate dehydrogenase

LDH-A - Lactate dehydrogenase A

LDH-B - Lactate dehydrogenase B

MGMT - *O*^{6}-methylguanine-DNA- methyltransferase

MMR - Mismatch repair

MTIC - methyltriazen-1-yl imidazole- 4-carboxamide

MTP - Mitochondrial transition pore

MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

MTZ - Mitozolomide

N^{3}-meA - O^{6}-meG, N^{7}-meG, and N^{3}-methyladenine

NICE - National Institute of Health and Care Excellence

NF-κβ - Nuclear factor-kappa β

PaTrin-2 - O^{6}-benzylguanine and O^{6}-(4-bromothenyl)guanine

PDH - Pyruvate dehydrogenase

PDK - Pyruvate dehydrogenase kinase

PET - Positron emission tomography

PKM2 - Pyruvate Kinase M2

PPP - Pentose phosphate pathway

p-PHD - Phosphorylated-PDH

RPMI 1640 - Roswell Park Memorial Institute 1640

RTK - Receptor tyrosine kinase

ROS - Reactive oxygen species*

Temodar/TMZ - Temozolomide

TCA - Tricarboxylic acid

TKIs - Tyrosine kinase inhibitors

TNF - Tumour necrosis factor

TNFα - Tumour necrosis factor α

shRNA - Short hairpin RNA

siRNA - small interfering RNA

WHO - World Health Organisation

CHAPTER ONE

Introduction

The term cancer refers to a range of diseases characterised by unregulated cell growth, invasion of surrounding tissue and the ability for cancer cells to spread from the primary site to secondary sites within the body in a process known as metastasis. Cancer is primarily a genetic disease and during the transition of normal cells to malignant state, they acquire a number of characteristics or 'hallmarks' (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). There are six essential alterations in cell biology that collectively dictate malignant growth and these are: (1) the ability to divide in the absence of growth factor stimulation, (2) the ability to divide in the presence of anti-growth signals, (3) the inability to undergo apoptosis, (4) the ability to maintain telomere length despite repeated cell divisions, (5) stimulation of angiogenesis, and (6) the ability to invade surrounding tissues and metastasize to other parts of the body (Figure 1).

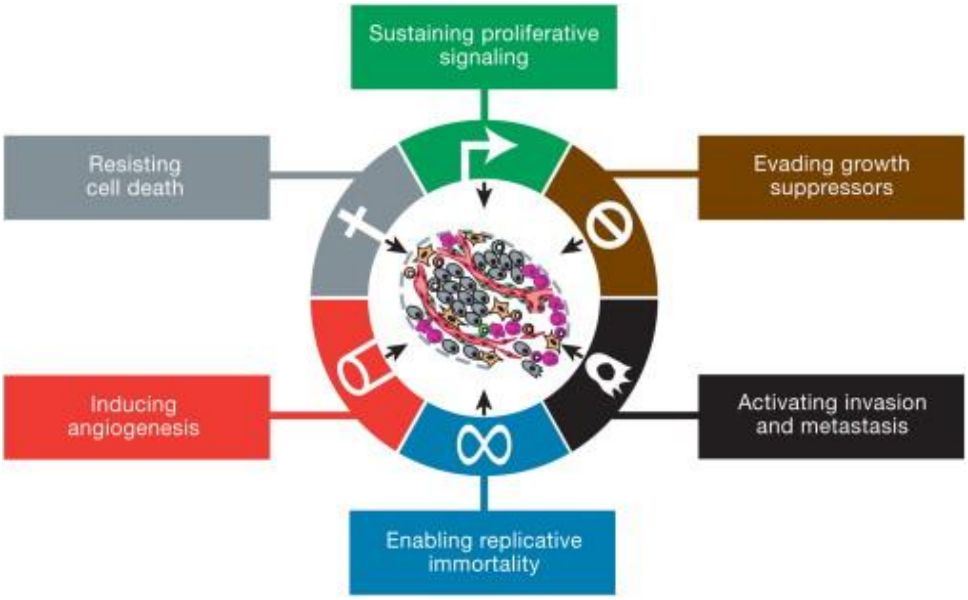


Figure 1.1: The six hallmarks of cancer as described by Hanahan and Weinburg in their landmark publication in 2000 (Hanahan and Weinberg, 2000).

In the more recent version, the authors revisited these hallmarks using information from transgenic animals and biochemical assays that did not exist a decade ago. Hanahan and Weinberg also added two categories in the new updated paper: "enabling characteristics" and "emerging hallmarks." The two enabling characteristics of cancer are tumour promoting inflammation and genetic instability and mutation, which assist cells in the transition from normal to oncogenic. The two emerging hallmarks; reprogramming of energy metabolism and evasion of the immune system have not been integrated into the canonical six because Hanahan and Weinberg remain unsure whether they are pervasive in all cancers (Hanahan and Weinberg, 2011).

In addition to providing a solid basis for cancer research, the hallmarks have served to identify certain cell functions that have become therapeutic targets. However, the utility of such attempts has been limited because tumour cells have demonstrated an ability to develop resistance to drugs that disrupt a single pathway. This adaptability of cancer cells suggests to Hanahan and Weinberg that simultaneous targeting of two or more hallmark pathways may be a more effective approach to therapy.

Cancer incidence and mortality

Cancer is a common disease accounting for 8.2 million deaths in 2012 according to the World Health Organisation (WHO). It is estimated that there will be 14.2 million new cases of cancer in 2012 worldwide with the incidence rates in males being greater than females by up to fourfold (Cancer, 2013). The most common cancers diagnosed were colorectal cancers, breast cancer, lung cancer and

prostate cancer and the most common causes of death from cancer were lung cancer, colorectal cancer, breast cancer and stomach cancer (Ferlay et al., 2010).

Regional and international differences in cancer incidence rates exist. In mainland Europe for example, the UK followed by France has the highest cancer incidence in males (426 per 100,000 and 385 per 100,000 respectively) and the UK followed by Demark has the highest incidence rate in females (347 per 100,000 and 328 per 100,000 respectively). In the UK 324,579 people were diagnosed with cancer in 2010 and this equated to 521 cases in every 100,000 people (males and females). In Europe the estimated number of cancer deaths in 2012 was 1.75 million (Ferlay et al., 2013), as compared with 1.256 million in 2007. According to statistics and figures within Europe for men, lung cancer accounts for the most deaths (182,080 deaths). In women, breast cancer accounted for the highest number of deaths (87,843 deaths) (Malvezzi et al., 2011). According to Cancer Research UK, cancer is responsible for more than one in four deaths in the UK and mortality rates are significantly higher in males than in females for all cancers combined. Therefore the need for prevention, early detection and new treatments is as obvious as it is imperative if the impact of cancer on the health and welfare of people and health service providers is to be reduced.

Current treatments for cancer

Following the diagnosis of a cancer patient, a team of practitioners are involved in the management and treatment of the disease. The main treatments used

today are surgery, radiation therapy and chemotherapy. Depending on pathological classification, staging and grading criteria, treatment regimes consist of either individual treatments or a combination of radiation therapy, chemotherapy and surgery or more. Surgery is the oldest form of cancer treatment and the most common of the treatments. There are different types of surgical procedures undertaken such as staging surgery, preventive surgery used to remove tissue that may become malignant, diagnostic surgery, and finally curative surgery. The latter is commonly used in the clinic to remove tumours confined to a single organ or site. In general, surgery is highly effective if the disease is localised and therefore early detection of tumours before they have metastasised is essential.

Almost half of all cancer patients receive some form of radiation therapy during the course of their treatment regime. Radiation therapy uses high energy radiation to induce DNA damage and shrink the tumour. Radiation therapy is effective in treating cancers that cannot be excised surgically and considerable technical advances in the field have led to greater focusing of the radiation field to the tumour mass. In general terms however, the effectiveness of radiotherapy depends upon being able to direct the radiation to the tumour mass and it is not particularly effective against disseminated tumours. In these cases, a systemic based therapy that has the ability to ‘search and destroy’ cancer cells anywhere in the body is required.

Chemotherapy is defined as the use of drugs to treat diseases such as cancer. Its origins in medicine stem back to the use of mustard gas in the First World War and the subsequent use of nitrogen mustard to induce remissions in patients with leukaemia (Ewig and Kohn, 1977). Today, the list of

chemotherapeutic drugs is extensive and can be broadly classified as classical cytotoxic and modern targeted therapeutics. The classical cytotoxic drugs target the process of cell replication via various mechanisms including DNA interactions (alkylation, intercalation, free radical damage induction etc.), DNA synthesis (the antimetabolites) and mitotic spindle poisons (Vinca alkaloids and Taxanes). Whilst these drugs can be extremely effective (Cisplatin based therapies are frequently curative against testicular cancer for example), their efficacy is constrained by toxicity towards normal tissues and the emergence of drug resistance (Hall and Tilby, 1992).

Throughout the history of cancer chemotherapy and drug development, there was an acknowledgement that more selective anti-cancer drugs were needed but the search for the 'magic bullet' was limited by lack of knowledge of the fundamental biological processes underpinning the cancer process. Following the revolution in molecular biology that occurred in the 1980's onwards, the biology of cancer is being unravelled and this has provided novel opportunities for drug discovery. In the 2011 version of the Hallmarks of Cancer review, Hanahan and Weinberg illustrate how this knowledge is leading to new therapeutic agents (Figure 1.2).

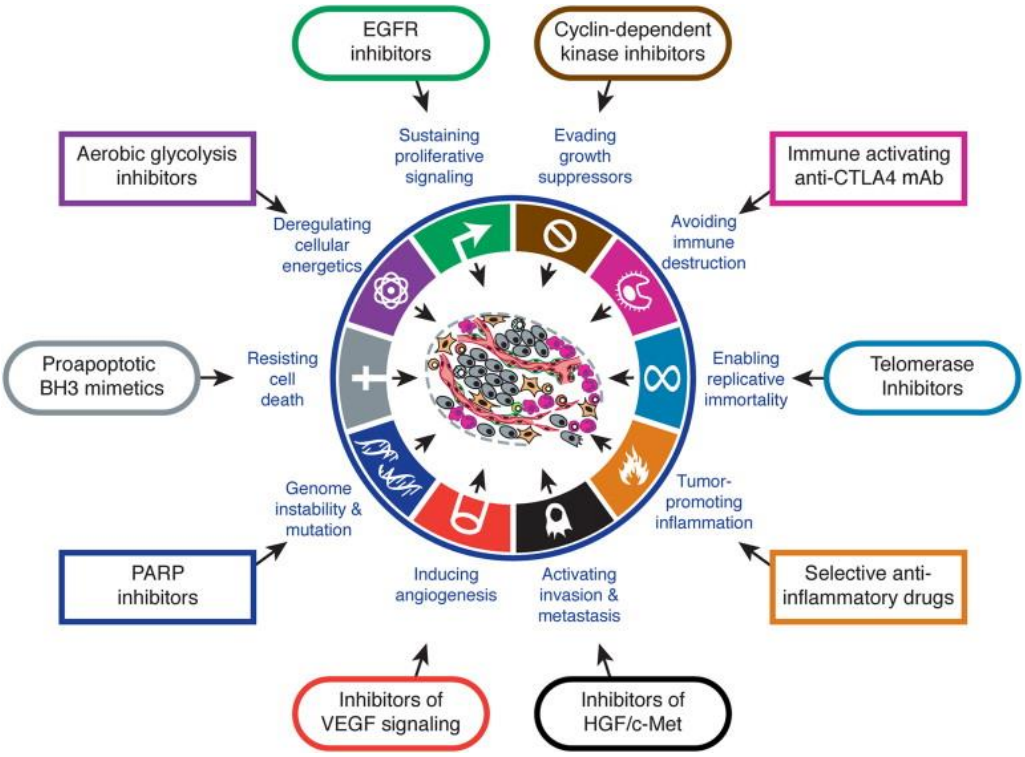


Figure 1.2: Understanding the biology of cancer is driving the development of new anti-cancer drugs. This figure taken from Hanahan and Weinberg (2011) illustrates some of the new, targeted therapeutic approaches being developed based on an understanding of the biology of cancer.

These new therapeutics are designed to interfere with specific oncogenic drivers and are classified as targeted or biological anti-cancer drugs. This new generation of anti-cancer drugs have been effective in many cases (e.g. glivec and chronic myelogenous leukaemia) but toxicity to normal tissues and the emergence of new drug resistance mechanisms mean that the promised hope of significant and dramatic breakthroughs in the war on cancer have not occurred. Progress has been made and the continued drive towards developing drugs to target oncogenic drivers (mutated proto-oncogenes in particular) offers hope for new therapies and the possibility of individualising chemotherapy based

on genomic analysis of tumours before and during treatment (Wu et al., 2006). A detailed review of targeted therapeutics is beyond the scope of this thesis and readers are referred to excellent reviews on this topic for information (Ciavarella et al., 2010; Lin; Wu et al., 2006). Suffice to say that whilst targeted therapies represent a significant advance in the way cancer is treated, their use is typically in combination with classical cytotoxic drugs and for the foreseeable future, cytotoxic drugs will still have an important role to play in the management of cancer. There remains a need to develop new anti-cancer drugs but equally, there is also a need to make better use of the drugs we already have. This thesis will address these issues in the form of two major chapters. The first looks at strategies designed to exploit the metabolic phenotype of cancers to enhance the activity of classical and targeted therapeutics and the second focuses predominantly on the drug Temozolomide and the evaluation of a series of novel analogues. The remainder of this introduction will focus on presenting the background to both these two topics.

Metabolic reprogramming of cancer and the Warburg effect

Louise Pasteur a leading scientist in his days described the phenomenon of “fermentation”, i.e. in order for cells to survive, cells switch from oxidative phosphorylation to glycolysis or fermentation in the absence of adequate oxygen. This phenotype was referred to as anaerobic glycolysis or the “Pasteur Effect” as described in figure 1.3A. Almost seven decades later Otto Warburg in the 1920's described the same phenomenon in tumour cells demonstrating that the fermentation process also occurs but this time, it occurred in the

presence of adequate oxygen. Today this aberrant metabolic feature, found in rapidly proliferating tumour cells is known as the “Warburg Effect” figure 1.3B (Weinhouse, 1976).

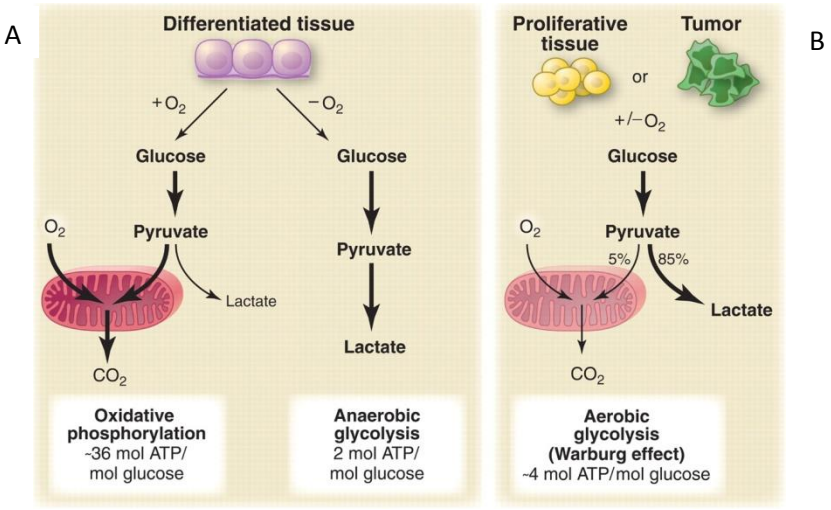


Figure 1.3: Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis (A) and aerobic glycolysis (the Warburg effect, B). Normal cells in the presence of oxygen or in the absence of oxygen metabolize glucose to pyruvate. In the presence of oxygen, normal cells will convert pyruvate to ATP via the Krebs cycle and oxidative phosphorylation and during anaerobic glycolysis (absence of oxygen) pyruvate is converted to lactate (A). In contrast to normal cells, cancer cells are metabolically re-programmed to convert glucose to lactate even in the presence of oxygen (B) (Vander Heiden et al., 2009).

Warburg observed that there was a correlation between the rates of glucose uptake, ATP production and the aggressiveness of tumour progression. Warburg originally assumed that aerobic glycolysis was a due to defects in mitochondrial respiration and he suggested that cancer might in fact be caused

by impaired mitochondrial metabolism (Gogvadze et al., 2008). It was later found that although there are defects in mitochondrial metabolism resulting in mitochondrial dysfunction (mutations in isocitrate dehydrogenase for example), the majority of tumours have essentially functional mitochondria but their activity is reprogrammed as a result of mutations in other pathways that drive the oncogenic process (Gatenby and Gillies, 2004a).

Even in the presence of an adequate oxygen supply, many tumours metabolize the majority of the glucose they take up through glycolysis. This glycolytic phenotype is due to cancer specific defects that impinge upon various stages of glycolysis, the Krebs cycle and oxidative phosphorylation. A term referred to as 'cancers sweet tooth' has been widely used to reflect cancers addiction to glucose and this has been exploited clinically to diagnose cancer and assess tumour response. The utilization of radiolabeled glucose analogue ^{18}Fluoro-deoxyglucose (^{18}FDG) in positron emission tomography (PET) combined with computed tomography (CT), plays an indispensable role in modern diagnostic oncology. An example of how FDG and PET imaging can assist detection of tumours is presented in figure 1.4. The success of metabolic imaging and the clear benefits achieved with FDG PET in the assessment of patients with cancer has had a significant impact on the management of clinical disease (Cintolo et al., 2013).

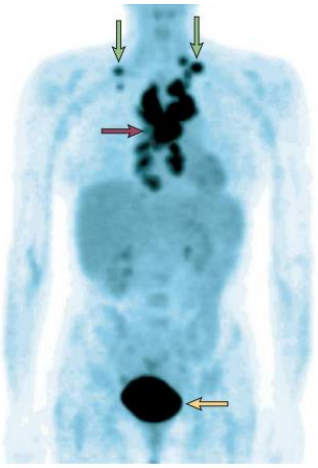


Figure 1.4: Positron-emission tomography (PET) imaging with ^{18}fluorodeoxyglucose (^{18}FdG) of a patient with lymphoma. The mediastinal nodes (purple arrow) and supraclavicular nodes (green arrows) show high uptake of ^{18}FdG showing that tumours in these nodes have high levels of ^{18}FdG uptake. The bladder (yellow arrow) also has high activity, because of excretion of the radionuclide (Gatenby and Gillies, 2004a).

Today this feature of cancer biology has reached the forefront of research and it was classified as an emerging hallmark of cancer (Hanahan and Weinberg, 2011). Key research questions have focused on understanding why cancer cells actively select this inefficient way of generating energy over oxidative phosphorylation and exploiting the opportunities it presents for therapeutic intervention. Selected issues of relevance to the work conducted in this thesis are reviewed in the following sections.

Mitochondria mediated apoptosis

Evasion of cell death or apoptosis is a hallmark of cancers and a major cause of treatment failure. It is now well established that many anticancer agents induce apoptosis (Kaufmann and Earnshaw, 2000), and that disruption of apoptotic pathways can influence treatment response (Schmitt and Lowe, 1999). Since agents with distinct primary targets can induce apoptosis through similar mechanisms, mutations or suppression of apoptotic pathways results in drug resistance (Dive and Hickman, 1991). An established example of this is p53. When activated following drug treatment, a series of events are initiated leading to activation of apoptosis. Loss of p53 function can diminish drug induced cell death thereby promoting resistance and survival (Wallace-Brodeur and Lowe, 1999).

The apoptotic process has been well characterised and it progresses through a series of biochemical reactions. The programme of cell death is controlled by apoptotic signalling networks and there are two major pathways for initiating apoptosis: an extrinsic pathway that responds to external signals such as the Fas ligand (FasL) and tumour necrosis factor α (TNFα), and an intrinsic pathway where a process of Ca^{2+} induced apoptosis is activated following an alteration in the normal operation of the mitochondria. These two pathways activate the caspase cascade responsible for carrying out the orderly cell death programme. The Bcl-2 superfamily, which has both pro- and anti-apoptotic members, plays a major role in regulating apoptosis, with much of its activity focused on the intrinsic pathway where mitochondrial induced apoptosis is an important element (Antonsson, 2004).

Apoptotic pathways

The intrinsic signalling pathway for programmed cell death involves non-receptor mediated intracellular signals, inducing activities in the mitochondria that initiate apoptosis. Stimuli for the intrinsic pathway include viral infections or damage to the cell by toxins, free radicals, or radiation. Damage to the cellular DNA can also induce the activation of the intrinsic pathway for programmed cell death. These stimuli induce changes in the inner mitochondrial membrane that result in the loss of transmembrane potential, causing the release of pro-apoptotic proteins into the cytosol. Pro-apoptotic proteins such as cytochrome *c* activate caspases that mediate the destruction of the cell through many pathways. These enzymes also translocate into the cellular nucleus, inducing DNA fragmentation, a hallmark of apoptosis. The regulation of pro-apoptotic events in the mitochondria occurs through activity of members of the Bcl-2 family of proteins and the tumour suppressor protein p53.

The extrinsic pathway begins when conditions in the extracellular environment determine that a cell must die. The pathway leading to apoptosis involves trans-membrane death receptors that are members of the tumour necrosis factor (TNF) receptor gene superfamily. Members of this receptor family bind extrinsic ligands and transduce intracellular signals, one such example is of caspases 2 and 8, these caspases recruit other signalling molecules such BID (BH3-interacting domain death agonist) which activates tBID (truncated BID). tBID recruits and activates Bax which aids the release of cytochrome *c* and this ultimately result in the destruction of the cell or apoptosis (figure 1.5). The signal transduction of the extrinsic pathway involves several caspases that are

proteases with specific cellular targets. Once activated, the caspases affect several cellular functions as part of a process that results in the death of the cell(s).

The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential. Therefore, research continues to focus on the elucidation and analysis of the cell cycle machinery and signalling pathways that control cell cycle arrest and apoptosis. The permeabilization of the mitochondrial outer membrane is a potent way of unleashing apoptosis inducing activators. Multiple apoptosis inducing factors and biochemical cascades converge on the mitochondria to cause the deregulation and permeabilization of the outer wall eventually inducing apoptosis. The mitochondria exerts both vital and lethal functions in physiological and pathological scenarios. On the one hand, mitochondria are indispensable for energy production and hence the survival of cells and on the other hand maintains crucial regulators of the intrinsic pathway of apoptosis controlled by the p53 tumour suppressor shown in figure 1.5. The p53 tumour suppressor protein can elicit apoptosis by up regulating the expression of pro-apoptotic Bax protein in response to sensing DNA damage; Bax in turn stimulates mitochondria to release cytochrome *c* (Bonnet et al., 2007). Two gatekeeper caspases, 8 and 9, are activated by death receptors such as FAS or by the cytochrome *c* released from mitochondria. The caspases trigger the activation of a dozen or more effector caspases that execute the cell death, via selective destruction of sub cellular structures and organelles and the genome. As the mitochondrion is a key regulator of cell death and for this reason mitochondrial functions are often altered in neoplasia, therefore mitochondrial oriented drug therapy represent a promising approach.

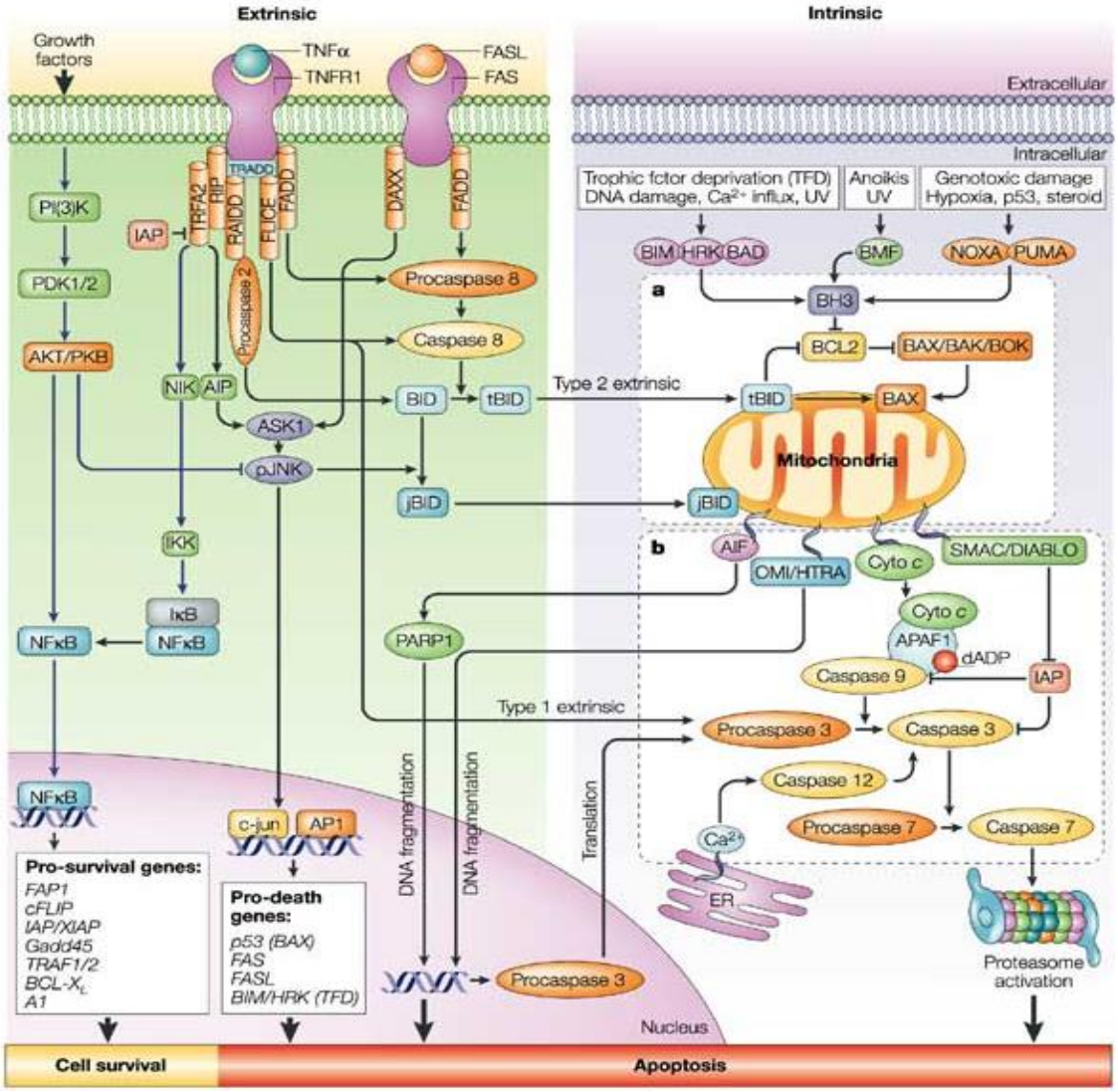


Figure 1.5: The extrinsic death pathway is initiated by binding of the extracellular ligands, TNFα (tumour necrosis factor α) or FASL (apoptosis antigen-1 ligand), to death receptors TNFR1 and FAS. TNFR1 activation leads to the formation of a TRADD-RIP-TRAF2 complex, which signals through the NF-κβ (nuclear factor κβ 3). The TRADD-FADD-FLICEZ complex activates precaspases 8 triggering apoptosis through caspases 3 or the cleavage of BID. The intrinsic apoptotic pathway is initiated at the mitochondrion where cytochrome *c* interacts with APAF1 (apoptosis protease activating factor 1) to recruit and activate caspases 9 forming apoptosomes which activate downstream caspases 3 and 7 (Benn and Woolf, 2004).

As stated previously, defects in the regulation of apoptosis are a hallmark of cancer and can contribute to the failure of chemotherapy. As both central metabolism and regulation of apoptosis converge on the mitochondria, one explanation for why cancer cells select for aerobic glycolysis is the avoidance of apoptosis via disturbance of mitochondrial physiology. To understand the link between metabolic reprogramming and apoptosis, some of the key features of the Warburg effect are described in the following paragraphs.

Two enzymes that play a prominent role in the glycolytic phenotype of cancer are pyruvate dehydrogenase kinase (PDK) and lactate dehydrogenase A and B (LDHA and LDHB) both of which are regulated by the transcription factor, hypoxia inducible factor 1 (HIF-1) (Shaw, 2006). PDK is a gate-keeping enzyme that regulates the flux of pyruvate into the mitochondria via controlling the activity of the pyruvate dehydrogenase (PDH) complex. In the presence of activated PDK, PDH is inhibited via phosphorylation of PDH, limiting the entry of pyruvate into the mitochondria, where the Kreb cycle and oxidative phosphorylation take place. In conjunction with suppression of PDH activity, a key feature of the Warburg effect is the production of lactate by LDH. LDH catalyses the conversion of pyruvate to lactate and expression of LDH is elevated in many cancers (Fantin et al., 2006). The combined effect of elevated LDH and PDK activity therefore is to reduce the amount of pyruvate entering the Krebs cycle. Due to no carbon entering the Krebs cycle via glycolysis and suppression of oxidative phosphorylation, the membrane potential across mitochondrial membranes is disturbed (Frezza and Gottlieb, 2009). Pro-apoptotic mediators, like cytochrome *c* and apoptosis inducing factor, are protected inside the mitochondria. When the voltage potential is altered and redox sensitive

mitochondrial transition pore (MTP) opens, apoptotic factors are released in the cytoplasm and induce apoptosis (Halestrap, 2005). Mitochondrial depolarisation and increased reactive oxygen species (ROS) are associated with opening of the MTP and mitochondrial membrane potential and ROS production are dependent on the flux of electrons down the electron transport chain (ETC), which in turn are dependent on the production of electron donors (NADH, $FADH_2$) from the Krebs' cycle. Suppressing the entry of pyruvate into the mitochondria by PDK and the inhibition of acetyl-CoA production, will suppress both Krebs' cycle and the ETC and therefore inhibiting the opening MTP and therefore inhibiting apoptosis (Michelakis et al., 2008).

Oncogenic regulation of the Warburg effect

Tumour suppressors act to maintain tissue homeostasis, that is, to control the number of cells reproduced and behaviour of cells in a particular tissue within an organism (Fridman and Lowe, 2003). Tumour suppressors regulate one or more processes that prevent aberrant proliferation (Vogelstein et al., 2000). The most extensively studied tumour suppressor is p53, and it acts in response to diverse forms of cellular stress to mediate a variety of anti-proliferative and apoptotic effects. Hence, p53 can be activated by DNA damage, hypoxia, or aberrant oncogene expression to promote cell-cycle checkpoints, DNA repair, cellular senescence, and apoptosis. As a consequence, disruption of p53 function promotes checkpoint defects, cellular immortalization, genomic instability, and inappropriate survival, allowing the continued proliferation and evolution of damaged cells. Given the profound proliferative advantage produced by loss of p53 function, it is not surprising that p53 is the most

commonly inactivated tumour suppressor gene in human cancer (Béroud and Soussi, 2003). In addition to its role in suppressing tumorigenesis, p53-dependent apoptosis contributes to chemotherapy induced cell death (Fridman and Lowe, 2003). This was first demonstrated in studies using oncogenically transformed cells treated *in vitro* and *in vivo*, and was subsequently extended to a variety of settings. Consistent with the potential role for p53 in modulating chemotherapy in human cancers, loss of p53 function was linked to chemoresistance in certain tumour types (Johnstone et al., 2002; Wallace-Brodeur and Lowe, 1999). These studies demonstrate the potential importance of apoptosis in cancer chemotherapy and initiated a link between cancer development and therapy. The intrinsic pathway centres on the mitochondria, which contain key apoptogenic factors such as cytochrome *c*. Major regulators of the intrinsic pathway are the pro- and anti-death members of the Bcl-2 family. These proteins reside at, or translocate to the mitochondria, controlling the release of the apoptotic inducing factors.

Whilst p53 is known to play a key role in regulating apoptosis and cellular metabolism, the oncogenic regulation of the Warburg effect is much more complex involving multiple oncogenes and tumour suppressor genes (Kim and Dang, 2006), details of which are reviewed in detail elsewhere (Kim and Dang, 2006; Koppenol et al., 2011). It is a widely held view that by switching their metabolism to aerobic glycolysis (which is driven by oncogenic events), cancer cells obtain a selection advantage by reducing their susceptibility to undergo apoptosis (Gogvadze et al., 2010). If this theory is correct, then interference with

the Warburg effect could have a major impact on ‘re-activating’ the apoptotic machinery making cells more susceptible to chemotherapy.

Opportunities to reactivate apoptosis by inhibition of the Warburg effect

1. Inhibition of LDH as a therapeutic strategy

LDH is a tetrameric enzyme comprised of two major subunits A and B. Five isozymes of LDH exist that can catalyse the conversion of pyruvate and lactate and the proportion of A and B subunits determines the direction of the reaction. All A (LDH-A) catalyses the conversion of pyruvate to lactate and all B (LDH-B) catalyses the reverse reaction (lactate to pyruvate). LDH-A is typically over expressed in cancers and this leads to the production of high levels of lactate in cancers. High LDH-A levels have been linked to poor prognosis in many cancers (Billiard et al., 2013) and reduction of LDH A levels in cancer cells by siRNA or shRNA reduces cellular proliferative and tumorigenic potential both *in vitro* and in xenograft models (Fantin et al., 2006; Wang et al., 2012).

The use of siRNA techniques to knock down LDH-A provides good evidence that LDH-A is a potential target for therapeutic intervention and several inhibitors of LDH-A have been explored (Granchi et al., 2011). One such inhibitor is Gossypol, a natural occurring compound originally isolated from cottonseed (Gossypium). It has been successfully used as a contraceptive drug for males in China where it was first discovered. The drug interferes with spermatogenesis, exerting a specific and reversible effect on developing sperm at concetrations of 2.5 -12.5μM (Wu, 1989). Gossypol has been shown to chelate zinc, bind arginine and lysine, and inactivate enzymes essential for

oxidative phosphorylation. Gossypol has been shown to inactivate LDH amongst other enzymes (Tuszynski and Cossu, 1984). Since gossypol exhibited such a broad spectrum of reactivity against metabolic enzymes such as LDH, it was investigated as a potential therapeutic compound against tumour cells that rely heavily on anaerobic metabolic pathways for production of energy. It was initially discovered by Tuszynski and Cossu (Tuszynski and Cossu, 1984) to have anticancer effects against several tumour cell lines grown in tissue culture, and in the most sensitive cell lines to gossypol, cell death or apoptosis was induced (Zhang et al., 2003). Gossypol competes with NADH for binding to lactate dehydrogenase which leads to competitive inhibition of lactate dehydrogenase activity (Yu et al., 2001). Gossypol has also demonstrated selective cytotoxic activity against cancer cells as opposed to normal cells or noncancerous cells (de Peyster and Wang, 1993; Volate et al., 2010) and these findings have been verified by independent research groups from different laboratories (de Peyster and Wang, 1993). Gossypol has been shown to inhibit the growth of various cancer cell lines *in vitro*, including breast, colon, prostate, and leukaemia. Inhibition of cytoplasmic and mitochondrial enzymes (including key enzymes for DNA replication and repair), uncoupling of oxidative phosphorylation, and depletion of cellular ATP are proposed mechanisms of action. In experimental models therefore, gossypol induces cytotoxic effects and is known to inhibit cell proliferation and prevent metastases in many different types of cancers (Stein et al., 1992; Volate et al., 2010).

The clinical testing of Gossypol began in the early 1970s in China, and to date the drug has been studied extensively in thousands of men (Wu, 1989). Clinical data from Gossypol in men have found Gossypol to be orally active and relatively

safe and effective but follow-up studies indicate that inhibition of spermatogenesis may continue following discontinuation in up to 50% of men after 2 years. Concerns regarding the lack of predictable, reversible effects have delayed the further clinical development of gossypol in Western countries (Coutinho, 2002). Nevertheless, phase I and II clinical trials are being conducted using gossypol in non-Hodgkin lymphomas, breast and prostate cancer. These results show limited promise in the clinic but its ultimate value as a clinical treatment requires further studies. Gossypol is nevertheless a good experimental tool to study the effects of pharmacologically inhibiting LDH in the preclinical setting.

2. Inhibition of PDK1 as a therapeutic strategy

PDK1, which is one of four family members, was identified as a direct HIF-1 target gene in hypoxic cells (Michelakis et al., 2008). PDK1 phosphorylates and inactivates the mitochondrial PDH complex. Suppression of PDH by PDK1 inhibits the conversion of pyruvate to acetyl-CoA, thereby attenuating mitochondrial function (apoptosis) and respiration (Kim et al., 2006). Because non-hypoxic stabilization of HIF through oncogenic events has been observed in many types of tumours (Semenza, 2003), PDK1 levels are also up-regulated in most tumours irrespective of oxygen tension. Elevation of PDK1 therefore prevents pyruvate from being metabolised by PDH resulting in increased lactate production which is a key feature of the Warburg effect (Kim and Dang, 2006).

Kim et al. (Kim et al., 2006) demonstrated that PDK1 mediated inhibition of the TCA cycle would result in increased glycolysis and ATP levels by shunting

pyruvate toward lactate production. By suppressing PDK1 expression by RNA interference, the growth of P493-6 (B cells) cells in hypoxia were impaired as compared to cells treated with control siRNA. The results suggested that PDK1 is necessary for the proliferation of P493-6 cells under hypoxic conditions and demonstrated that it is a potential target for therapeutic intervention.

Wun Lu et al. (Lu et al., 2008) found that PDK1 and PDK3 were up regulated under hypoxic conditions, and were interested to determine the roles of PDK1 and PDK3 on HIF1α-induced drug resistance. Transient transfection of HeLa cell with siRNA against and ablation of PDK1 resulted in increased cell death under hypoxia. Furthermore double knockdown of both PDK1 and PDK3 caused more cell death under hypoxia suggesting an additive effect on cell survival. When these cells were treated with cisplatin or paclitaxel, PDK1 deficient cells exerted cell death compared with PDK3 knockdown cells. Simultaneously, knockdown of PDK1 and PDK3 resulted in further increasing susceptibility to drug killing. Together, this research provides compelling evidence to demonstrate that PDK1 and PDK3 played an additive effect in HIF1α-induced drug resistance (Kim et al., 2006; Wang et al., 2004).

A small molecule of 150 Daltons called dichloroacetate (DCA) is a known inhibitor of PDK1, and is often used to treat lactic acidosis in patients with mitochondrial defects (Stacpoole et al., 1994). DCA inhibits PDK1 resulting in the reduction of lactic acid production by reducing glycolysis and increasing oxidative phosphorylation (Ko and Allalunis-Turner, 2009). Inhibition of PDK1 by DCA caused a shift in metabolism resulting in the reactivation of oxidative phosphorylation and altered mitochondrial membrane potential. The

depolarization of the mitochondrial membrane potential resulted in the release of the apoptotic effector cytochrome *c* and increased apoptosis (Michelakis et al., 2008). An increase in reactive oxygen species generated by oxidative phosphorylation up-regulates the voltage gated K^+ channel, leading to potassium ion efflux and caspase activation inducing apoptosis. The reversal of metabolic reprogramming of cancer cells by DCA has been shown to also reverse cell resistance to apoptosis mediated by the mitochondria. DCA returns the membrane potential of cancer cells towards the levels of the healthy cells, without affecting the mitochondria of healthy cells as illustrated in figure 1.6. This suggests that forcing cancer cells to respire aerobically can reinstate mitochondrial oxidative phosphorylation and counteract the reduction of apoptosis caused by the Warburg effect.

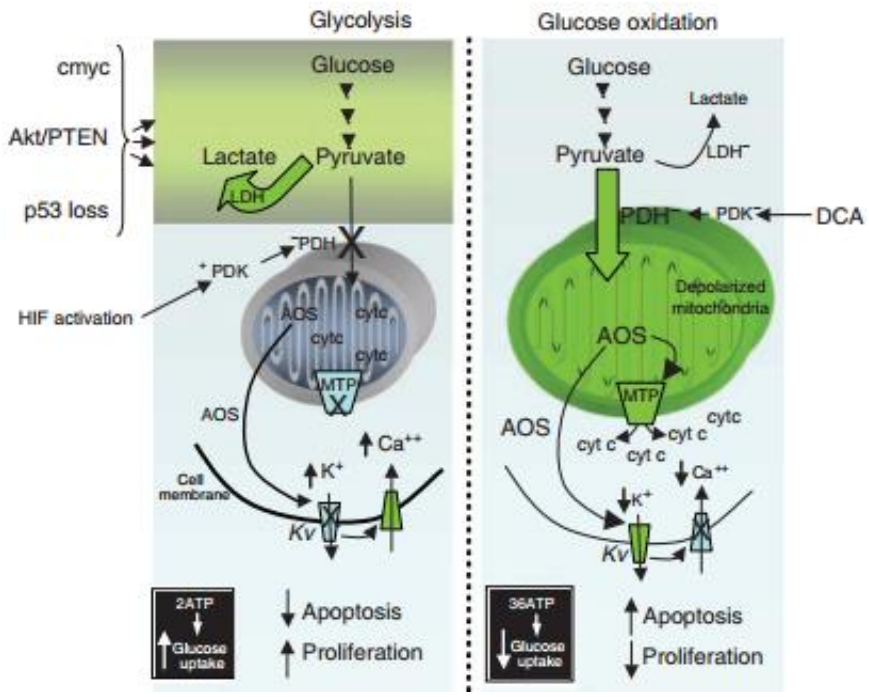


Figure 1.6: In cancer cells mitochondrial glucose oxidation is suppressed and cytoplasmic glycolysis is favoured. The Kv channels (blue) are blocked and cannot allow the exit of K^+ out of the cell (panel A) and an increase entry of Ca^{++} contributes to the inhibition of apoptosis and a hyperpolarised state within the mitochondria. PDH activation by DCA or LDH inhibition promotes oxidative phosphorylation by increasing the entry of pyruvate into the mitochondria and Krebs cycle. Consequently, apoptosis increases cell proliferation and tumour growth decreases (Michelakis et al., 2008). *K+: Potassium ions, MTP: Mitochondrial transition pore, AOS: Activated oxygen species, Kv: Potassium voltage*

In vitro, DCA inhibition of PDK was observed at low concentration of 10–250μM in a dose dependent manner (Stacpoole, 1989). DCA administered at 35–50 mg kg^{-1} lead to a decrease in lactate levels (> 60%) (Parolin et al., 2000).

DCA inhibits its own metabolism and the half-life increases with subsequent doses (Stacpoole et al., 2006). However, there is a plateau of this effect and DCA serum levels do not continue to rise with chronic use (Mori et al., 2004). DCA has been used for the past 40 years in children and adults with diverse disease states including healthy volunteers.

In 2006 the first randomised control trial were carried out with DCA in congenital mitochondrial diseases. The first, a blinded placebo-controlled study was performed with oral DCA administered at 25 $mg\,kg^{-1}\,day^{-1}$ in 30 patients (Kaufmann et al., 2006) with MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes). Following this a second study was undertaken another randomised placebo controlled double blinded study with 21 children suffering from congenital lactic acidosis treated with DCA orally at 25 $mg\,kg^{-1}\,day^{-1}$ for 6 months demonstrated mild peripheral neuropathy (Stacpoole et al., 2006). No other toxicities were reported. Peripheral neuropathy is a frequent complication induced by taxane, platinum and vinca-alkaloid chemotherapies, it also can be induced by DCA but may depend on whether cancer patients have prior or concurrent neurotoxic therapy (Michelakis et al., 2008).

3. Combination of inhibitors of the Warburg effect with anti-cancer drugs

Whilst the evaluation of inhibitors of cancer cell metabolism as single agents is ongoing, it is important to state that most chemotherapeutics will be used in combination with other cytotoxic or targeted therapeutics. In the context of targeting cancer metabolism and the potential for re-activating the apoptotic

phenotype of cancers by altering mitochondrial physiology, combination therapy is particularly relevant. Several studies have demonstrated that modulating LDH and/or PDK can increase the efficacy of cytotoxic drugs (Papandreou et al., 2011), little is known about the effects metabolic inhibitors would have on targeted therapeutics. Before discussing this in more detail, some background to targeted therapeutics is provided in the following paragraphs.

As illustrated in figure 1.2 (Hanahan and Weinberg linking hallmarks to drugs) modern anti-cancer drugs are designed to target key features of tumour biology. One example of this early success of directed targeting is tamoxifen, which targets the estrogen receptor, a key driver of a significant number of breast cancers. Such success gave rise to new agents which targets proteins specific to cancer (Levitzki, 2013). Aberrant cell signalling is a hallmark of cancer and considerable efforts have been made to interfere with this process. Tyrosine kinase inhibitors (TKIs) represent one class of drugs that have been developed and many are approved for clinical use (Paul and Mukhopadhyay, 2004). Thus, targeted therapies represent a new addition to the arsenal of agents used to treat cancer and this approach has already lead to beneficial clinical effects. A detailed review on targeted therapeutics is beyond the scope of this thesis and readers are referred to excellent reviews on the topic for further details (Ciavarella et al., 2010; Sawyers, 2004).

Combination therapy involves using two or more chemotherapy drugs against cancer cells to achieve a better or improved therapeutic outcome. Combination chemotherapy was first established in the 1960s when scientists wondered whether the approach to treating tuberculosis using a combination of antibiotics

to reduce the risk of resistance would work for the treatment of cancer (Mayer and Janoff, 2007). Chemotherapeutic agents are often combined to enhance efficacy by reducing recurrence and delaying the emergence of resistant tumours. Combination therapy is now an integral part of cancer chemotherapy treatments and the benefits provided have demonstrated great therapeutic efficacy. An example of the potential benefits from combination studies are presented in the treatment of breast cancer in the paper published by Miles and his research team who found that combination and sequential therapy both have their place in the treatment of metastatic breast cancer (Miles et al., 2002). Newer drug combinations, such as paclitaxel/trastuzumab or capecitabine/docetaxel, show survival advantages over single-agent therapy and have manageable safety profiles and potentially reduce the risk of toxicity, which may improve quality of life (Bull et al., 1978; Norton, 1997).

Following DCA treatment, it has been described and shown that DCA can potentiate the activity of a number of conventional cytotoxic drugs such as Temozolomide (Michelakis et al., 2010), Doxorubicin (Heshe et al., 2011) and platinum based drugs (Olszewski et al., 2010). Little is known however about the ability of DCA to potentiate the activity of targeted therapeutics such as TKIs. Because of the link between oncogenic pathways and the Warburg effect, it is conceivable that inhibition of the Warburg effect could impact upon the activity of TKIs. Similarly, it is possible that the reverse situation could also apply in that TKI's could potentiate the activity of inhibitors of aerobic glycolysis. As no studies have investigated combinations of TKI inhibitors and inhibitors of glycolysis, this thesis will address this issue. The first aim of the thesis therefore is to determine

whether or not manipulation of the Warburg effect using inhibitors of PDK1 and LDH can enhance the activity of clinically used TKIs.

Glutamine 'addiction' and the promotion of cell proliferation.

In addition to the avoidance of apoptosis, it is now believed that switching metabolism to aerobic glycolysis supports the proliferation of cancer cells which is a key hallmark of cancer biology (Buchheit et al., 2012). In order to maintain cell proliferation, cells require the building blocks needed to replicate (i.e. lipids, nucleic acids etc.). This need requires the cell to make anabolic metabolites. In normal tissues that are rapidly proliferating, the glycolytic pathway and Krebs cycle are diverted from energy production (catabolism) to biosynthetic pathways (anabolism). Full details of the metabolic reprogramming that promotes cell proliferation has been extensively reviewed elsewhere (Hanahan and Weinberg, 2011; Le et al., 2012) but the remainder of this section will focus on the glutamine dependency of tumours and how this promotes cell proliferation.

Historically, during research in to the nutritional requirements of cell lines growing *in vitro*, Eagle observed that the glutamine consumption rate of many of the cell lines exceeded the consumption of any other amino acid by tenfold (Harry, 1955). Cell lines he studied could not proliferate in the absence of exogenous glutamine and many could not maintain their viability in the absence of glutamine (Kaadige et al., 2009). Glutamine has therefore been recognized for a long time to play a unique role in the metabolism of proliferating cells. Most tumours consume and utilize glutamine at much higher rates than other amino acids (Daye and Wellen, 2012). Although glutamine is a non-essential amino

acid in normal and non-dividing tissue, it is essential for the proliferation of most cells and the viability of some cancer cells that have become addicted to glutamine. Glutamine metabolism contributes to the ability of cancer cells to continuously grow and proliferate by supporting ATP production and biosynthesis of proteins, lipids, and nucleic acids (DeBerardinis and Cheng, 2010; Wise and Thompson, 2010). Recent investigations into glutamine metabolism have greatly expanded our understanding of the role and regulation of glutamine metabolism in cancer (DeBerardinis and Cheng, 2010; Reinert et al., 2006). Glutamine plays several important metabolic roles in the cell, it serves as a carbon source for energy production, contributes carbon and nitrogen to biosynthetic reactions and co-operates with glucose uptake to drive the Warburg effect, all of which are summarised in figure 1.7.

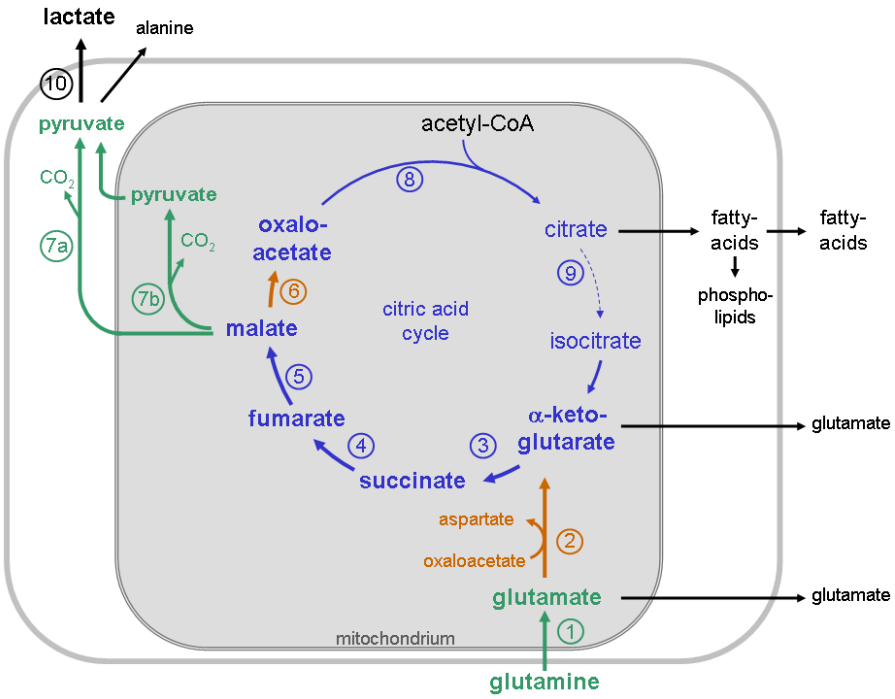


Figure 1.7: Glutamine metabolism in cancer cells and the glutaminolytic pathway. Steps in the Krebs cycle are in blue. The malate aspartate shuttle appears in orange and the green color represents enzymes overexpressed in tumors. The numbers denote the following enzymes: 1 = glutaminase, 2 = Glutamine oxaloacetate transaminase (glutamate dehydrogenase can also convert glutamate to α-keto-glutarate), 3 = α-keto-glutarate dehydrogenase, 4 = succinate dehydrogenase, 5 = fumarase, 6 = malate dehydrogenase, 7a = cytosolic malic enzyme, 7b = mitochondrial malic enzyme, 8 = citrate synthase, 9 = aconitase, 10 = lactate dehydrogenase (Wise and Thompson, 2010).

The synthesis of fatty acids is essential for cell replication and one route involves the removal of citrate from the TCA cycle where it is converted to acetyl CoA in the cytosol and utilised to drive fatty acid and phospholipid synthesis (Le et al., 2012). Because the TCA cycle is now interrupted, alternative sources of α-keto-glutarate are required to maintain the supply of oxaloacetate required to drive

the next 'cycle' of the TCA (Figure 1.6). Cancer cells achieve this objective by importing glutamine and converting this to α-keto-glutarate that enters the TCA. This truncated form of the TCA is a key feature of tumour cell metabolism and it has provided novel opportunities for therapeutic intervention, details of which are reviewed in detail elsewhere (Frezza and Gottlieb, 2009). These include the development of inhibitors of key enzymes involved in glutamine metabolism and several are in preclinical development.

An alternative approach is to employ a glutamine depletion strategy on the basis that reduction or removal of external sources of glutamine would slow down tumour growth. Various glutaminases have been identified and are being explored but one avenue of research involves the potential use of L-asparaginase. An essential nutrient in rapidly growing cancer cells is asparagine, which is required to proceed through the G1 cycle of cell division. Asparagine is important in protein, DNA, and RNA synthesis. The amino acid, L-asparagine, is a nutritional requirement of both normal and cancer cells. Unlike normal cells, however, certain leukemic cells cannot synthesize asparagine and must consequently rely on an external supply in the plasma and tissues. The administration of the enzyme, L-asparaginase, destroys this free source of asparagine, starving and killing certain cancer cells (Pieters et al., 2011). In conjunction with being able to remove asparagine, it is reported that L-asparaginase also has glutaminase activity (Cairns et al., 2011). There are several commercial sources of L-asparaginases, each of which is isolated from different bacteria. The version from *Erwinia chrysanthemi* (Erwinase or Kidrolase) is believed to have significantly enhanced glutaminase activity

compared to L-asparaginase isolated from *Eschericia coli* (Narta et al., 2007). Whilst these therapeutics are not approved for use in solid tumours, the fact that they can deplete glutamine warrants further investigation as a possible adjunct to conventional targeted or classical chemotherapeutic agents.

Cells with enhanced expression of Myc oncoproteins are particularly sensitive to glutamine deprivation, which induces depletion of TCA cycle intermediates, resulting in a depression of ATP levels and a delay in growth, and apoptosis. Myc drives glutamine uptake and catabolism by activating the expression of genes involved in glutamine metabolism, including *GLS* (glutaminase) and *SLC1A5* (glutamine transported) (Gao et al., 2009). Silencing glutaminase mimicked some of the effects of glutamine deprivation, including growth suppression in Myc-expressing cells and tumours (Cheng et al., 2011). Understanding glutamines role in metabolic pathways, and how cancer cells benefit from glutamine addiction can be informative, and used in clinical oncology as a potential therapeutic strategy. Glutamine is a highly versatile nutrient whose metabolism has implications for tumour biology and may provide novel therapeutics opportunities. Key questions remain concerning the level of glutamine depletion that cells have to be exposed to in order to reduce cell growth and what happens to cells when glutamine levels return to normal after a period of depletion. These and other issues represent the second major objective of this thesis and they will be addressed in chapter two.

Development of novel analogues of Temozolomide

As stated previously, the search for new and more effective therapies to treat cancer remains a major objective in the War on Cancer. New chemotherapies can be developed along target orientated principles or by developing analogues of successful drugs that have been engineered to provide solutions to problems encountered by the parent drug. Temozolomide (TMZ) is one such example and the third chapter of this thesis will present studies designed to evaluate novel analogues of TMZ. Initially however, the pharmacological properties of TMZ are reviewed below.

Temozolomide; mechanisms of action and resistance

TMZ is an orally available, mono functional alkylating pro-drug that has demonstrated efficacy in the treatment of a variety of solid tumours, including primary malignant brain tumours and metastatic melanoma (O'Reilly et al., 1993). TMZ has certain advantages over many existing alkylating agents because of its small molecular weight; this allows TMZ to efficiently cross the blood brain barrier (Agarwala and Kirkwood, 2000). Thus, TMZ is effective against primary brain tumours; TMZ can be administered orally providing it with a unique bioavailable profile and is also associated with a low incidence of severe adverse toxicity.

TMZ achieves its cytotoxic effect mainly by methylating the O^6 position of guanine. This leads to damaged DNA and to apoptosis (O'Reilly et al., 1993). TMZ spontaneously hydrolyses to methyltriazen-1-yl imidazole- 4-carboxamide (MTIC) as shown in figure 1.8. MTIC then degrades into methyldiazonium ion, which is the active methylating species. TMZ can methylate the DNA at N^7

guanine, O^3 adenine, and O^6 guanine but TMZ is most effective when it methylates DNA at O^6 position of guanine (Beier et al., 2008). This adduct can be removed by the DNA repair protein O^6-methylguanine-DNA-methyltransferase (MGMT) therefore TMZ displays its highest efficacy against tumours lacking MGMT expression due to a methylated MGMT promoter (Hegi et al., 2005).

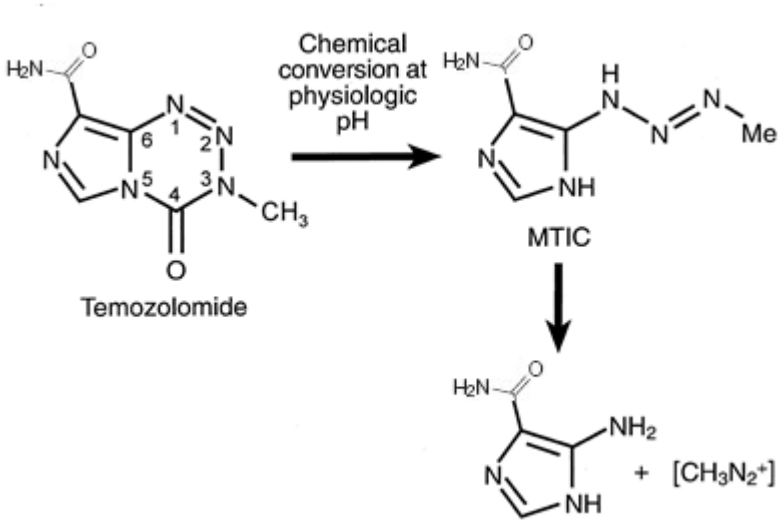


Figure 1.8: A schematic diagram for the conversion of Temozolomide to MTIC. It undergoes rapid chemical conversion in the systemic circulation at physiological pH to the active compound MTIC (Friedman et al., 2000).

TMZ has few side effects but like most chemotherapy drugs it does induce toxicity due to its unselective nature. Common side effects of TMZ include, constipation, fatigue, and headache, loss of appetite, low blood platelet count, nausea, vomiting, and weakness. Some of the less common side effects include anaemia, low white blood cell count, hair loss, rash, and convulsions, swelling

in the arms or legs, difficulty sleeping, and/or a tingling or burning feeling. Some of the more major side effects are myelosuppression, a condition in which bone marrow activity is decreased, resulting in fewer red blood cells, white blood cells, and platelets (Friedman et al., 2000).

There are several mechanisms of resistance to alkylating agents, these include defects in DNA mismatch repair (MMR), increased excision repair, and increased enzymatic removal of alkyl groups (Zhang et al., 2012). One of the key enzymes responsible for removing methyl groups from O^6-methylguanine is O^6-alkylguanine transferase (AGT), also known as MGMT. Over expression of MGMT can lead to resistance to the cytotoxic effects of TMZ (Agarwala and Kirkwood, 2004; Lee et al., 1994). However, TMZ is able to deplete the levels of MGMT in various cell types, thus reducing the potential for drug resistance (Newlands et al., 1992). TMZ induced cell death is inhibited by cells expressing MGMT. Therefore cells which express MGMT are immune from the effect of TMZ. Conversely, cells that lack MGMT expression are sensitive to TMZ.

Mutagenic lesions which are not repaired by MGMT are recognized by the post-replication MMR. MMR corrects DNA mismatches generated during DNA replication, thereby preventing mutations from being passed on in dividing cells. The MMR system is required for cell cycle arrest and/or programmed cell death (apoptosis) in response to certain types of DNA damage such as TMZ and other alkylating agents (Li, 2007). MMR is not functional in many tumours, either as a result of a mutation in one of the MMR genes, or because of an epigenetic silencing due to methylation in the *hMLH1* gene (Aarnio et al., 1999; Hoeijmakers, 2001). Defective MMR pathway and increased expression of

MGMT can lead to the tumour resistance to TMZ. This deficiency in the MMR pathway results in a failure to recognize and repair the O^6-MG adducts produced by TMZ (Friedman et al., 2000). DNA replication continues past the O^6-MG adducts without cell cycle arrest or apoptosis providing immunity to cancer cells from TMZ. MGMT can be successfully inactivated by free guanine base derivatives, with alkyl groups at the O^6 position, which act as ‘‘pseudosubstrates.’’ O^6-benzylguanine and O^6-(4-bromothenyl)guanine (PaTrin-2) have been identified as the most promising MGMT in-activators (Liu and Gerson, 2006). Compared to Temozolomide used as a single agent, the combination PaTrin2/Temozolomide has been shown to significantly increase tumour growth inhibition in human melanoma xenografts. PaTrin2 and O6-benzylguanine have recently entered phase II clinical trials. A key question for future research is the development of analogues of TMZ that circumvent these resistance mechanisms. A series of novel analogues of TMZ were synthesised by Dr Richard Wheelhouse (School of Pharmacy, University of Bradford) and the initial evaluation of these compounds against a panel of cell lines that have been characterised for MMR and MGMT is presented in chapter 3 of this thesis.

Aims and objectives

Whilst considerable progress has been made in reducing mortality in some forms of cancer, mortality rates for other cancers remain high and there is a need to develop novel therapeutic approaches. Understanding the biology of the disease and the pharmacology of drugs used to treat cancer is driving forward the development of novel anti-cancer drugs and novel strategies to make existing drugs work better. Within this context, the purpose of this thesis is to address three key issues;

The first is whether or not the Warburg effect can be pharmacologically manipulated to enhance the activity of targeted anti-cancer drugs. Specifically, inhibitors of LDH-A and PDK1 will be evaluated in a panel of cell lines and combination studies *in vitro* will be conducted using a series of clinically approved tyrosine kinase inhibitors.

The second objective is to determine what effect glutamine depletion strategies have on the viability and response of cells *in vitro*. These studies will address key issue's relating to the 'depth' of glutamine depletion required to induce cellular responses and whether or not the effects of glutamine depletion are reversible once glutamine levels return to normal.

Finally, TMZ is a multi-billion dollar drug used to treat glioblastoma. Whilst effect, its efficacy is compromised by drug resistance mechanisms including deficiencies in MMR and over-expression of MGMT. Novel analogues of TMZ will be evaluated with the aim of identifying second generation compounds that retain the desirable properties of TMZ but have improved properties in terms of

circumvention of the drug resistance mechanisms that have reduced the efficacy of TMZ.

CHAPTER TWO

Introduction

Targeting the glycolytic phenotype and glutamine addiction in cancer cells.

The Warburg effect is now considered to be an emerging hallmark of cancer (Hanahan and Weinberg, 2000) and is defined by the increased dependence of cancer cells on glucose metabolism even in the presence of oxygen. As normal cells transform into cancerous cells, the normal processes for energy production of glycolysis, the Krebs cycle and oxidative phosphorylation are reprogrammed and cells become reliant on glucose metabolism and aerobic glycolysis (Gatenby and Gillies, 2004b). Aerobic glycolysis rapidly generates ATP and diverts carbon from glucose into precursors for the synthesis of nucleotides, proteins, and lipids to support the growth of the rapidly dividing population of cancer cells. As a consequence of this switch, glucose is preferentially catabolized to lactate, rather than fully metabolized via mitochondrial oxidative phosphorylation.

As indicated in chapter 1 and summarised in figure 2.1, there are two major molecules that are catabolized in substantial quantities in cancer cells. These are glucose and glutamine. Glucose and glutamine supply the majority of the carbon, nitrogen, free energy and reducing equivalents necessary to support cell growth and division in proliferating cells. Inhibiting LDH activity impairs cell proliferation (Dang et al., 2009). Furthermore, highly proliferative cells need to produce excess lipid, nucleotide, and amino acids for the creation of new biomass. Therefore excess glucose is then diverted through the pentose phosphate pathway (PPP) to create nucleotides (Kim and Dang, 2006).

Glutamine supplies nitrogen for nucleotide and hexosamine synthesis while glutamate is the nitrogen donor for the synthesis of many nonessential amino acids. It is now widely appreciated that many of the signalling pathways that promote oncogenesis also reprogram glutamine metabolism, and in many cells the dependence on glutamine is absolute, a condition termed glutamine addiction (Wise and Thompson, 2010).

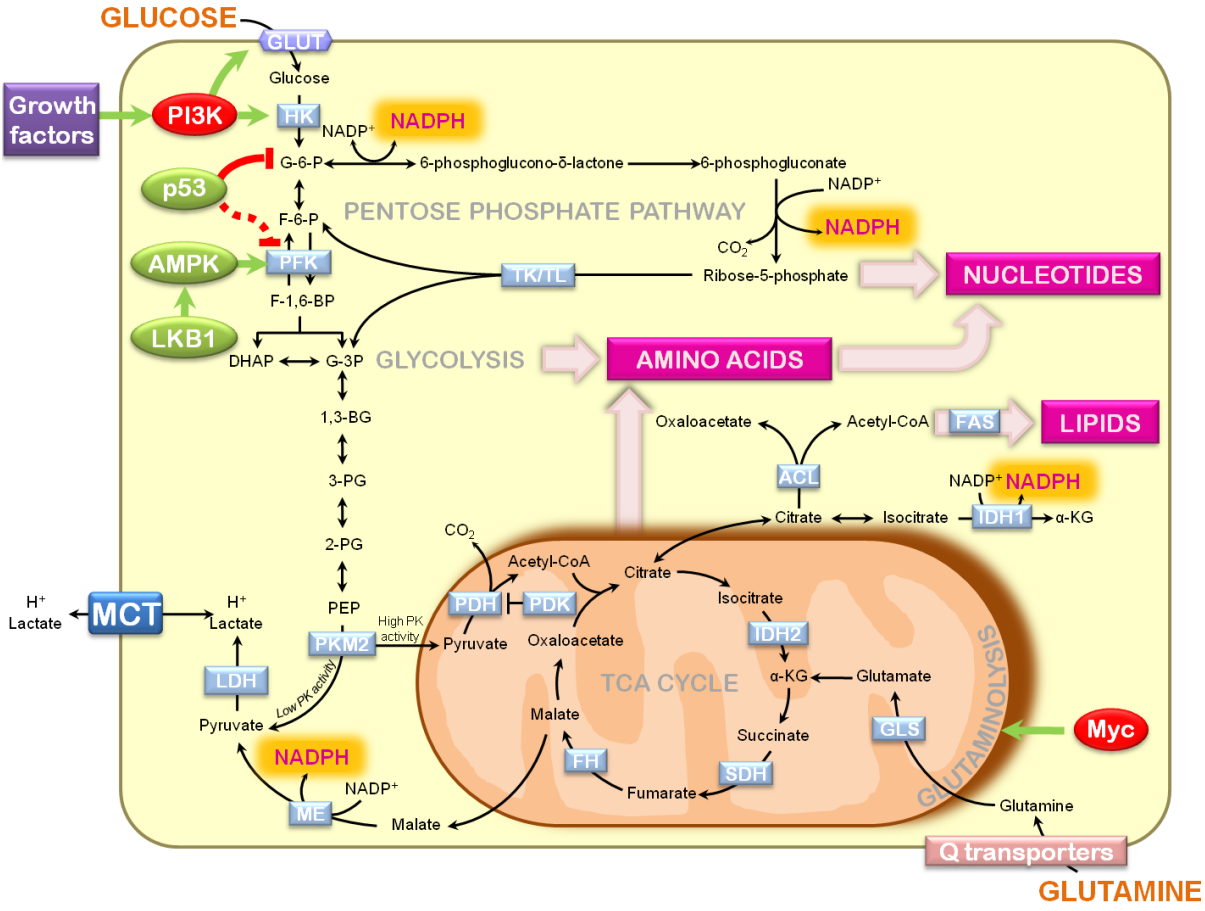


Figure 2.1: The role of glucose and glutamine in cancer cells as a result of metabolic adaptations imposed by oncogenes and tumour suppressors. As a consequence of such metabolic reprogramming is that glucose can be utilised for the production of ATP via glycolysis resulting in lactate production and glutamine utilized for the synthesis of amino acids, lipids and nucleotides, which are essential for proliferating cells. Such metabolic adaptations are central to tumour progression in turn providing the opportunity for therapeutic targets such as glutamine, LDH and PDK (Wise and Thompson, 2010).

Oncogenic events in cancer drive the switch from oxidative phosphorylation to aerobic glycolysis and lactate production by inducing the expression and activation of several glycolytic enzymes. To begin with the aberrant PI3K/AKT

signalling induces the expression and cell surface expression of glucose transporters (GLUT1 and GLUT4) (Gottlob et al., 2001). Followed by the transcriptional oncoproteins MYC and HIF-1α induce the expression of several glycolytic enzymes including LDH A. In addition, MYC enhances glutamine catabolism by inducing the transcription of the glutamine transporters (ASCT2; also known as SLC1a5) and by suppressing miR-23a/b, which normally blocks glutaminase translation (Gao et al., 2009). Studies have detected an up-regulation of high affinity glutamine transporters in cancer (Fuchs and Bode, 2005). One primary example, SLC1A5 (ASCT2), is a direct target of the Myc oncoprotein, and is up-regulated in a host of cancers. In the search for Myc-regulated mitochondrial proteins, Gao et al. found that glutaminase protein levels were significantly up-regulated in Myc-overexpressing cells. Myc-induced metabolic reprogramming triggered cellular dependency on exogenous glutamine as a source of carbon for the maintenance of the mitochondrial membrane potential and macromolecular synthesis prolonging survival (Gao et al., 2009). Higher glutaminase expression results in increased glutamine uptake and catabolism. MYC also promotes the production of the M2 form of pyruvate kinase (PKM2), a normally embryonic isoform of pyruvate kinase that is catalytically inefficient and favours aerobic glycolysis (Christofk et al., 2008). Finally, coupling of glycolysis to oxidative phosphorylation is disabled by MYC or HIF-1α directed induction of pyruvate dehydrogenase (PDH) via pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates PDH (Kim et al., 2006).

Glutamine has an important role in cell growth and energy metabolism. Glutaminolysis, consists of two steps: the first is catalysed by glutaminase and

converts glutamine to glutamate, whereas the second is catalyzed by glutamate dehydrogenase (GDH) and converts glutamate to *α*-ketoglutarate (*α*-KG). There are two types of glutaminase in mammalian cells, kidney-type glutaminase (GLS1) and liver-type glutaminase (GLS2) (DeBerardinis et al., 2008). Metabolic flux experiments show that cancer cells exhibiting Warburg like metabolism do not stop utilizing the TCA cycle instead these cells come to rely on glutamine as the carbon source for the TCA cycle (DeBerardinis et al., 2007). Many cancer cells rely on glutamate anaplerosis to refill the TCA cycle. Glutamate enters the TCA cycle via its conversion to α-ketoglutarate by the actions of either transaminases or glutamate dehydrogenase (DeBerardinis and Cheng, 2010). Malate and citrate created by the TCA cycle are then available for further biosynthetic pathways. This allows the intermediates generated by the TCA cycle to feed other biosynthetic pathways as precursors. Therefore, cancer cells are dependent on glutamine to maintain the TCA cycle (Kamata et al., 2007). This indicates that elevated glutaminolysis is linked to drug resistance.

Rationale and Aims

As described in chapter 1 and elaborated above, the switch to aerobic glycolysis may be beneficial to tumour cells as it contributes towards the avoidance of apoptosis, a classical hallmark of cancer. By reactivating oxidative phosphorylation, it is possible in some cases to reactivate the apoptotic phenotype making cells more susceptible to therapeutic drugs. Inhibiting LDH and/or PDK1 by Gossypol or DCA respectively may lead to an increase of pyruvate in the mitochondria promoting glucose oxidation, reactivating apoptosis and decreasing proliferation and tumour growth. Inhibitors of key enzymes involved in the Warburg effect (e.g. DCA) have been shown to potentiate the activity of certain drugs (e.g. Temozolomide (Michelakis et al., 2010), Doxorubicin (Heshe et al., 2011) and platinum based drugs (Olszewski et al., 2010).

However, no studies have investigated whether DCA can influence the activity of TK inhibitors. In addition to targeting the glycolytic phenotype, glutamine depletion strategies may also be effective in reducing the cancer burden. A variety of human cancer cell lines have shown sensitivity to glutamine starvation, including pancreatic cancer, glioblastoma multiforme, acute myelogenous leukemia, and small cell lung cancer (Wise and Thompson, 2010). It is not known however whether glutamine depletion has to be complete to cause cell death or what effect replenishment of the media with glutamine has on cell survival. The specific aims of the work conducted in this chapter are listed below:

1. To determine the activity of inhibitors of LDH A (Gossypol) and PDK1 (DCA) against a panel of cell lines *in vitro*.

2. To determine whether the activity of TKIs are influenced by inhibition of PDK1 and LDHA

3. To determine how much glutamine needs to be depleted in order to reduce the growth or kill tumour cells *in vitro*.

Methods and Materials

Materials

All chemicals were purchased from Sigma Aldrich (Poole, United Kingdom) unless stated otherwise. All tissue culture consumables were purchased from Costar (Amsterdam, Netherlands).

Cell Lines and Maintenance

Isogenic human colon colorectal carcinoma cell lines HCT116 $p53^{+/+}$ (wild type) and HCT116 $p53^{-/-}$ (null type) cell lines were obtained from the Vogelstein laboratory (Johns Hopkins Kimmel Cancer Centre, Baltimore, USA) and were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium, supplemented with 10% foetal bovine serum, sodium pyruvate (1mM) and L-Glutamine (2mM). To study the effects of different media on cell growth, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were also maintained in Dulbecco's Modified Eagle Medium (DMEM) Glutamax II (purchased from GIBCO, UK) supplemented with 10% foetal bovine serum and sodium pyruvate (1mM). These cells were maintained at 37˚C in a 5% CO_2 humidified atmosphere in an incubator until required for experimental use.

Routine maintenance and Sub-culturing of cell lines

Cells were kept in exponential growth by regular sub-culturing and were not allowed to reach more than approximately 70% confluence (estimated by visual inspection of flasks). First the medium from the flask was removed and the adherent cells growing at the bottom of the flask were washed twice using Hanks

Balanced Salt Solution (HBSS) followed by the addition of trypsin (1-3ml of 0.25% trypsin EDTA) to the cell layer. Following incubation for up to 3 minutes at room temperature or at 37^{o}C until the cells had detached, 10ml of complete RPMI 1640 culture medium or DMEM culture medium was then added to the cells to stop the enzymatic activity of trypsin. The cell suspension was subsequently centrifuged for 5 minutes at 800*g*. After obtaining a cell pellet, the supernatant was discarded and re-suspended in 20ml of complete RPMI 1640 or DMEM culture medium. Complete RPMI 1640 or DMEM medium was added to new T75 flasks and 1ml of cell suspension was added in to the T75 flasks. Flasks were then incubated in 5% CO_2 and at 37˚C.

Cell counting using a haemocytometer

After cleaning the haemocytometer with 70% ethanol, the glass cover slip was fixed in to place over the etched grids and pressed down until Newton's rings were visible. A small volume of cell suspension was pipetted on to the haemocytometer (approximately 10µl) at the point where the cover slip and the haemocytometer met. The fix between the cover slip and the haemocytometer aided uptake via capillary action of the cell suspension. As illustrated below in figure 2.2 cells were counted in 5 squares (labelled in red). Cells on the left and the top edges of the grid were counted (in green) whereas cells that covered the right side of the chamber and bottom edges of the grid were not counted (in red). Using the cell count from five individual counts the mean was calculated. The mean cell number is multiplied by x10^4 to give the number of cells/ml. Cells were counted using an inverted microscope at X10 objective magnification.

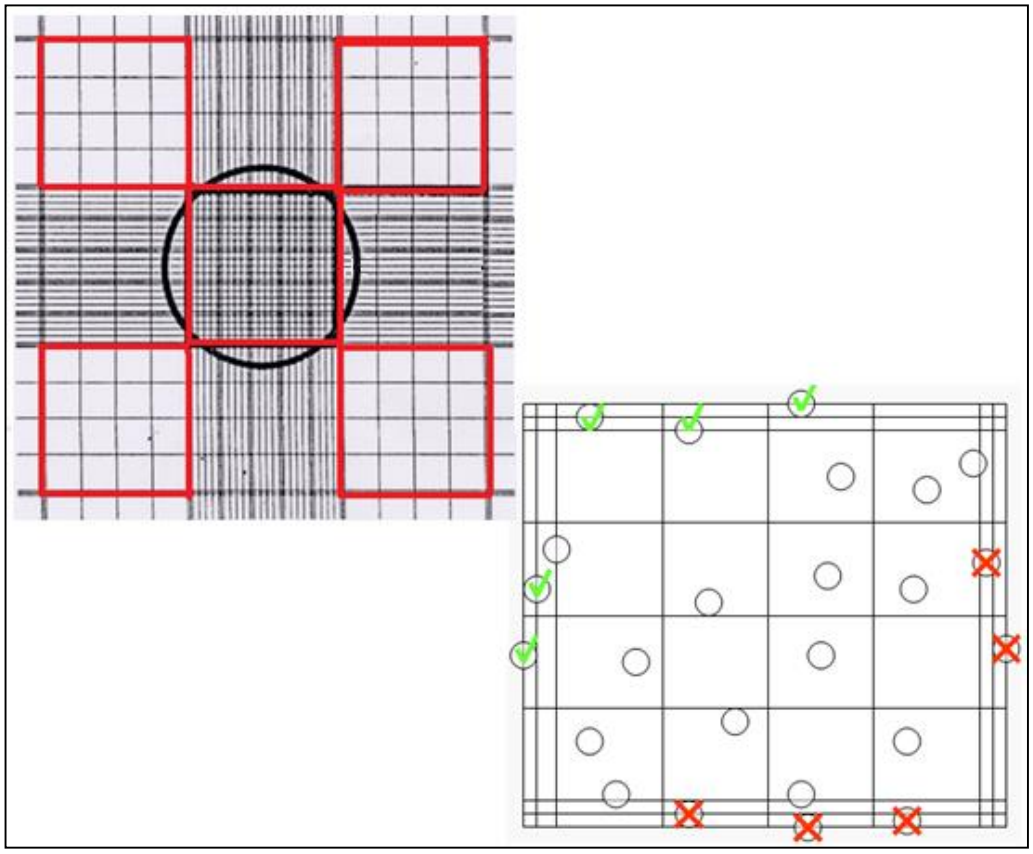


Figure 2.2: A schematic diagram of a haemocytometer and the five chambers (in red) used to count cells. Top left (A) represents the five chambers used for counting cells and the bottom right (B) represents the process used to count cells that fall on the edge of the haemocytometer grids. Cells which have been marked with a green tick are cells included in the count and cells which are marked with a red cross are excluded from the count.

Chemosensitivity studies: Experimental conditions for drug exposure

The response of cells to cytotoxic drugs was determined using the MTT assay (van Meerloo et al., 2011). Details of the cytotoxic agents used are presented in subsequent sections below but the generic protocol is described here. Briefly, a $2x10^4$ cells/ml cell suspension was made in a final volume of 10ml. Cells were

plated into a 96 well plate at a density of $2x10^3$ cells per well as illustrated in figure 2.3. Following overnight incubation (at 37˚C) the culture medium was removed from the wells and replaced by medium containing varying concentrations of drug (8 wells per drug concentration). Column 1 rows A-H were used for the blank (RPMI 1640 or DMEM only) and column 2 rows A-H were used for the control (cells plus RPMI 1640 or DMEM medium). The remaining columns (3 to 12) contained compounds at a range of concentrations. The 96 well plates were incubated for further 6 days (144 hours at 37˚C in a 5% CO_2) before carrying out the MTT assay. All experiments were done in triplicate. For experiments that involved a 1 hour drug incubation, 96 well plates were prepared as described above and following the 1 hour exposure, cells were washed twice with HBSS (200 µl per well). After washing the wells, fresh medium (200µl) was added to all the wells and cells were incubated (at 37˚C) for a further four days before carrying out the MTT assay. All experiments were done in triplicate.

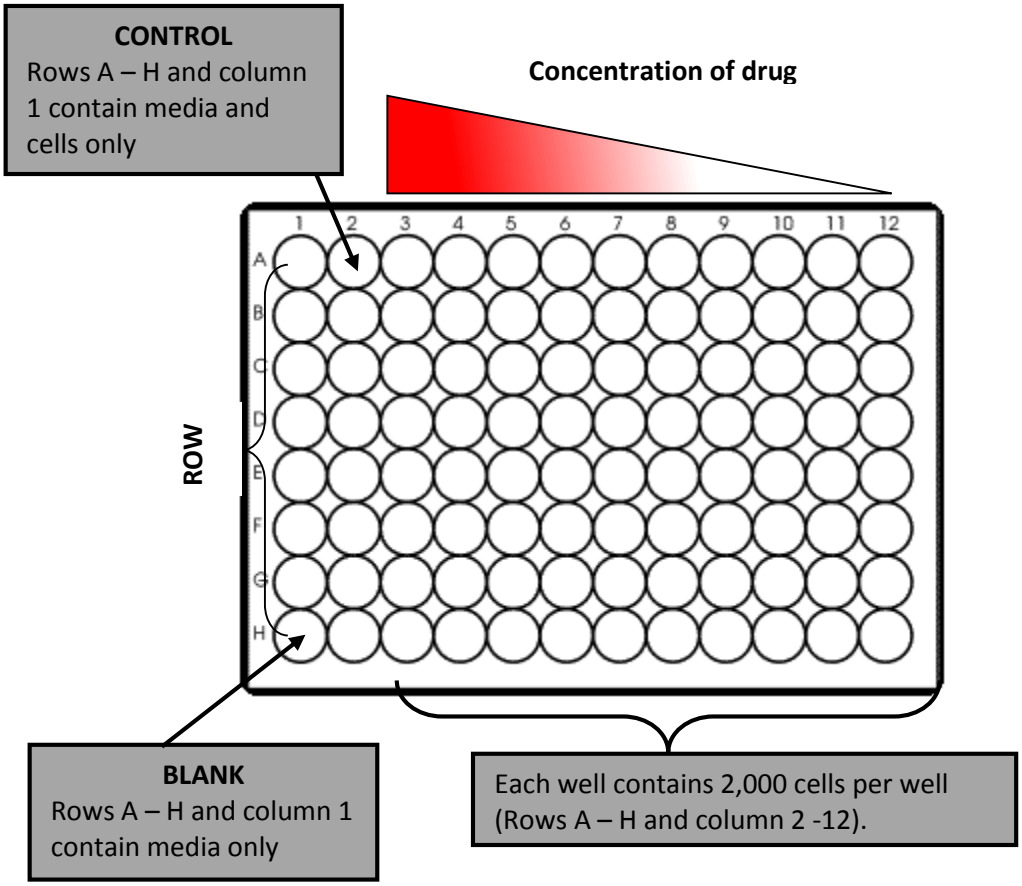


Figure 2.3: A schematic diagram of a 96 well plate describing the setup of the plate for chemosensitivity testing. After seeding the plate with cells it was incubated overnight to allow the cells to adhere to the bottom of the wells before the drug was added. The triangle (in red) represents the drug concentration gradient; column 3 contained the highest concentration of drug whereas column 12 contained the lowest drug concentration. Columns 1 and 2 represents the blank (no cells, media only) and the control (containing cells and the drug vehicle only) respectively.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is a yellow, water soluble tetrazolium salt that is reduced by viable cells to purple, water

insoluble formazan crystals. The reduction takes place in the mitochondria of viable cells by enzymes such as succinate dehydrogenase (van Meerloo et al., 2011). To each well of the 96 well plate(s), 20µl of 5mg/ml MTT solution is added and the plate(s) are incubated at 37°C for 4 hours. After the incubation, the content of the wells was removed leaving behind the formazan crystals at the base of the well. The formazan crystals were solubilized by the addition of 150µl of dimethyl sulfoxide (DMSO). Once the crystals dissolved, the absorbance of the solution was determined at 540nm using a Multiskan Ex-microplate reader (Thermo Scientific, UK) with a 540nm filter. The mean absorbance of the blank was subtracted from all other mean values in order to obtain the true absorbance. Percentage cell survival was determined according to the following equation:

$$\%\ \text{CELL SURVIVAL} = \left\{ \frac{\text{Absorbance of treated lanes}}{\text{Absorbance of control lanes}} \right\} \times 100$$

Dose response curves were generated by plotting % cell survival against drug concentration and the IC_{50} was determined. IC_{50} is defined as the concentration of drug required to reduce cell survival by 50%.

Measuring the Response of Cells to Dichloroacetate, Gossypol, Bortezomib and Tyrosine Kinase Inhibitors

The response of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ following continuous exposure to gossypol was determined using the MTT assay as described above. Gossypol was dissolved in complete RPMI 1640 culture medium at a stock concentration of 100mM, aliquoted and stored at -20°C until required for

experiments. HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were exposed to a range of drug concentrations (100mM to 0.195mM in two fold dilutions) for 6 days before cell survival was determined using the MTT assay.

The response of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ following continuous exposure to DCA was determined using the MTT assay as described above. DCA was dissolved in complete RPMI 1640 culture medium and was made up fresh on the day of the experiment. Initially, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were exposed to DCA (concentrations ranged from 100mM to 0.195mM using a 2 fold dilution) for 6 days. Subsequent studies used increased concentrations of DCA (ranging from 0.976mM to 500mM using a 2 fold dilution) to ensure a full dose response curve was obtained. DCA was made up with complete RPMI 1640 and DMEM for the respective experiments. The response of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ maintained in complete RPMI 1640 and DMEM was determined to see whether differences in the culture medium influenced chemosensitivity.

The response of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells following continuous exposure to TKIs and Bortezomib was determined using the MTT assay as described above. HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were exposed to TKIs and Bortezomib for 6 days. TKI concentrations ranged from 0.156μM to 80μM using a 2-fold dilution and Bortezomib ranged from 0.302nM to 156nM and the concentration of DCA ranged from 0.976mM to 500mM. All dilution were made using a 2-fold dilution. All compounds were dissolved in DMSO at the stock concentrations indicated in table 1. Working concentrations of TKIs and

Bortezomib were all made up with complete RPMI 1640 to ensure that the final DMSO concentration was < 0.1% in all cases.

Name	Molecular weight	Stock concentration	Solvent
Dasatinib	488.01	100mM	DMSO
Imatinib	589.71	100mM	DMSO
Gefitinib	446.9	100mM	DMSO
Masitinib	498.65	100mM	DMSO
Bortezomib	384.24	100mM	DMSO
Sorafenib	464.82	100mM	DMSO
Vandetanib	475.35	100mM	DMSO
Nilotinib	529.52	75mM	DMSO
Sunitinib	398.47	50mM	DMSO

Table 1: Table of each individual drugs used in experiments to measure the cytotoxicity against HCT cell lines and the basic information regarding each drug. All compounds were obtained from LC Laboratories (Woburn, USA).

Combination chemosensitivity studies: DCA in combination with TKIs and Bortezomib

Based on the studies above, the IC_{50} for the individual TKIs and Bortezomib were used for the combination study. The diluent was made up of complete RPMI 1640 culture medium plus the TKI or Bortezomib at their respective IC_{50} values. The diluent was used to make up the highest concentration of DCA

(500mM) and subsequent dilutions to provide a range of DCA concentrations. Cells were exposed to drug combinations for 6 days prior to analysis of cell survival using the MTT assay.

For the combination study there were variations to the layout of the plate. This was the inclusion of an additional control. The first of the two controls was column 2 rows A-H (cells plus medium) the second was column 3 rows A-H (cells plus medium with TKI/Bortezomib only). The second control was used to calculate % cell survival when evaluating the effectiveness of the combination experiments.

Statistical analysis

Statistical analysis was carried out on data for Bortezomib and TKI alone and in the presence of DCA. Student –T test was used to find out whether the data has differences or similarities in respect to the experimental groups. A value of less than 0.05 (P value <0.05) was considered statistically significant.

Western Blot Analysis for PDK1 inhibition by DCA

The purpose of the experiments was to detect PDH, PDK1 and the phosphorylated form of PDH (p-PDH) in the absence and presence of DCA. In the absence of DCA the following should be detected PDH, PDK1 and p-PDH whereas in the presence of DCA, PDK1 should be inhibited therefore the

detection of PDH should be detected whereas the detection of the p-PDH should be reduced.

Preparing cells for Western Blot analysis

HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were seeded in T25 flasks at $1x10^5$ in 8ml of complete RPMI 1640 culture medium. A total of 6 T25 flasks were prepared for each cell line, flask one was used for the control, the remaining five flasks were labelled 1-5 and were incubated for five days at 37^{o}C and 5% CO_2. The concentration of DCA ranged from 31.25mM to 500mM (5 concentrations made with a 2 fold dilution factor) and cells were exposed to DCA for 1hour.

Following drug incubation, the supernatant in each flask was collected and placed in to labelled universal tubes (this was done to ensure none of the detached cells in suspension were lost). Adherent cells growing at the bottom of the flask were washed with HBSS twice followed by trypsinisation; 1ml of trypsin (0.25% trypsin EDTA) was added to the cell layer and incubated for up to 3 minutes at room temperature until the cells had detached. Phosphate buffer saline (PBS) solution was added (5ml) to the cells to dilute and reduce the enzymatic activity of trypsin. The cell suspension was centrifuged (800*g* for 5 min), following the centrifugation the supernatant was discarded keeping the cell pellet, which was washed with cold PBS (5ml) twice.

Proteins were extracted from the cell pellet by adding lysis buffer (0.5mM EDTA, 0.5mM EGTA, 5mM NaCl, 1mM Tris-HCl (pH 7.5), 1mM dithiothreitol, protease inhibitor mixture (protease inhibitor cocktail 2 and 3, Sigma Aldrich, Poole, United Kingdom) and 10% Triton X-100 in deionised H_2O (3 times the volume of the pellet) followed by sonication (2 x 10 second bursts using settings of 5 cycles

and 50% power (US-70 from Phillip Harris Scientific (Lichfield, UK)). The lysates were centrifuged for 10 minute at 10,000*g* and 4°C, the supernatant was collected for Western blot analysis and the protein concentration was calculated by using the Bradford assay.

Measurement of protein concentration using the Bradford Assay

The total protein concentration in each sample was measured using the Bradford assay (Bradford, 1976), which relies on the linear relationship between absorption and the amount of Coomassie dye bound to the proteins present in the lysate. The standard curve was established by preparing serial dilutions of bovine serum albumin (BSA) ranging from 0.0312mg/ml to 1mg/ml (BSA stock solution 1mg/ml). The blank (distilled water) and protein standards (50µl) were added to 1,450ml of Bradford reagent, mixed and incubated for 10 minutes at room temperature. The absorbance at 595nm was measured using a MultiScan Spectrum spectrophotometer (Thermo Scientific, UK). All calibration and unknown samples were assayed in triplicate. The concentration of protein in test samples was determined from the linear part of the standard curve (using the equation derived from linear regression analysis). Samples where absorbance values fell outside the linear range of the calibration curve were diluted to ensure absorbance values fell within the linear range. Typically, the cell lysate samples were diluted by a factor of 20 in order to obtain absorbance values within the linear range. The dilution factor was used to determine the final protein concentration in samples.

Western Blot Analysis

Western blots were performed by running 50µl of protein extracts suspended in extraction buffer on a 12% SDS polyacrylamide gel and transferred to nitrocellulose membranes by standard procedures described below. The apparatus used for all electrophoresis experiments was the Biorad mini trans-well blot system and the first step in the Western blotting process was to prepare the gel; to make a 12% resolving gel the following reagents were added:

Resolving gel (12%)			
Components	**5ml = 1 gel**	**10ml = 2 gels**	**Storage**
deionised H_2O	1600µl	3300µl	Room temperature
30% acrylamide	2000µl	4000µl	Fridge 4^oC
1.5M Tris (pH8.8)	1300µl	2500µl	Room temperature
10% SDS	50µl	100µl	Room temperature
10% APS	50µl	100µl	Freezer -20 ^oC
TEMED	2µl	4µl	Room temperature

*APS= Ammonium persulfate, *SDS= sodium dodecyl sulfate, *TEMED= Tetramethylethylenediamine

Once the resolving gel was been poured a thin layer of iso-butanol was added on top of the newly poured gel. After the gel has polymerised, the iso-butanol was removed and any excess iso-butanol was removed via washing with dH_2O and blotting paper. The stacking gel (see below) was poured on top of the running gel and the combs were carefully inserted avoiding air bubbles. The

combs provide wells for the sample and were left in for up to 15 minutes to all the stacking gel to polymerise.

Stacking gel			
Components	**1ml = 1 gel**	**2ml = 2 gels**	**Storage**
deionised H_2O	680µl	1400µl	Room temperature
30% acrylamide mix	170µl	330µl	Fridge 4^oC
1.0M Tris (pH6.8)	130µl	250µl	Room temperature
10% SDS	10µl	20µl	Room temperature
10% APS	10µl	20µl	Freezer -20 ^oC
TEMED	1µl	2µl	Room temperature

Once polymerised, the gels were placed into the tank and immersed in electrophoresis buffer (25mM Tris base, 192mM glycine, 0.1% SDS and pH 8.3). The combs were removed from the gel and the wells were washed with electrophoresis buffer to remove any un-polymerised acrylamide.

The ladder (5ul of the ladder to 15ul of sample buffer (2x)) was prepared, followed by the blank (10ul of sample buffer plus 10ul of PBS) and the samples (x amount of cell sample to equal parts of sample buffer (2x) and the remaining volume added was deionised water (final volume 20ul)). These were all incubated for 15 minutes at 90^oC. The ladder (protein molecular weight standards used as molecular weight markers) was added to the first well

followed by the samples to subsequent wells and the gels were run at 60 volts for 15-20 minutes, allowing the samples to run through the stacking gel at the same rate passing through in to the running gel. Once the samples reached the running gel the voltage was increased to 120volts until the ladder reached the bottom. The final step was to remove the gels and prepare them for the transfer process.

Before removing gels for transfer process, 500ml of the transfer buffer was prepared (48mM Tris base, 39mM glycine, 0.04% SDS, 20% methanol) and placed in a large container. A small volume of transfer buffer was removed and placed in small container which was used to soak the blotting paper (x2) and white pads (x2).

Nitrocellulose paper was cut to the size of the gel(s) and soaked in to deionised water for one minute. During this time the gels were removed from the glass plates housing the gels and prepared for the transfer process. The transfer cassettes were opened (black side down) and placed in the large container containing transfer buffer, the white pads were placed on to the cassette (black side) followed by blotting paper. The gel was placed on top of the blotting paper then the nitrocellulose paper was placed over the gel. Finally placing the second piece of blotting paper over the nitrocellulose paper followed by the white pad carefully removing any air bubbles. The transfer cassette was closed by bringing the clear side down and locking both sides in to place. The cassette was then placed in to the cassette holder (the black side facing the panel which was black). The cassette(s) holders were placed in to the tank, which was then filled with transfer buffer followed by placing the lid over the top and sealing the tank. The transfer process was ready to begin and the tank was placed in to the

container and packed with ice. The current was set at 300mA for 1 hour, after which the transfer process was stopped and the cassette holder(s) were removed. The cassette was opened removing the layers to retrieve the nitrocellulose paper which was then incubated with blocking buffer (5ml of 5% milk in PBS/Tween) for 1 hour at room temperature and then 4^{o}C overnight.

After the overnight incubation the nitrocellulose membrane was further exposed to 5% non-fat milk or 5% BSA (5% BSA was used for the detection of PDH-pSer293) in PBS/Tween (0.05%) for 1 hour at room temperature. After the blocking step, membranes were washed with 5ml PBS/Tween (0.05%) twice and then incubated with the respective primary antibody for 1 hour at room temperature followed by overnight incubation on the shaker at 4^{o}C. The primary antibodies used were:

- PDH E1 phosphoserine 293, (Rabbit - Polyclonal) 1:3000 (1µl of anti-body in 3,000 µl in 5% non-fat milk) (Novus Biologicals – Cambridge, UK)
- PDK-1 (Rabbit - Polyclonal), 1:2,500 (1µl of anti-body in 2,500 µl in 5% non-fat milk (Novus Biologicals – Cambridge, UK)
- PDH E1 (Mouse – Monoclonal), 1:5000 (1µl of anti-body in 5,000 µl in PBS/Tween (0.05%)) (Invitrogen – Paisley, UK)
- β-Actin (Rabbit - Polyclonal), 1:10,000 (1µl of anti-body in 10,000 µl in 5% non-fat milk) (Abcam – Cambridge, UK)

After exposing the nitrocellulose membrane to the primary antibody the membranes was washed (5% BSA) and the next step was to expose the membrane to the secondary antibody. Secondary antibody (anti-rabbit or mouse depending on Ab) at the desired dilution (1:35000) in the 5% blocking buffer was

added followed by incubating the membrane with the secondary antibody on shaker for 1 hour at room temperature. The secondary antibody was removed, replaced with 5ml of PBS/TWEEN (0.05%) (15 minutes) and placed on the shaker at room temperature. This step was repeated a further two times. Commencing the second wash the nitrocellulose membrane was placed in a clean container and incubated with 600µl ECL reagent (300µl of ECL reagent A to 300µl of ECL reagent B) for up to a minute. Carefully removing the nitrocellulose membrane followed by removing excess ECL reagent, the nitrocellulose membrane was then placed in to a developing cassette. The nitrocellulose membrane was placed between two clear pieces of film.

To develop the nitrocellulose membrane, the following was carried out in the dark room. The red light was turned on and measures were taken to minimise exposure to light, the developing film was cut approximately the same size of the membrane, and placed over the nitrocellulose membrane followed by closing the developing cassette for up to 2 minutes. After the 2 minute incubation the developing film was removed and placed in a tray with the developer (50ml developing solution to 450ml tap water) for up to 3 minutes. The developing film was washed with water to remove any developer. Placing the membrane in the fixing solution (75ml fixing solution to 425ml tap water) for 2 minutes then rinsing the developing film in water thoroughly to remove all traces of fixing solution, leaving it to dry. All experiments were carried out in triplicates.

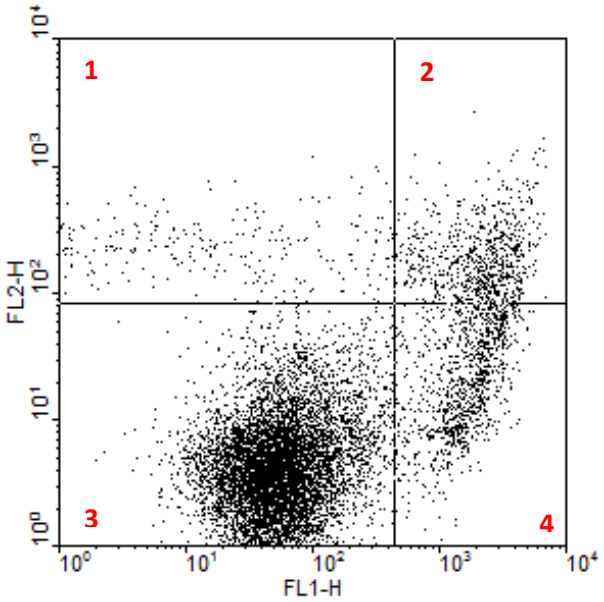
Detecting apoptosis induced by Etoposide, DCA and Gossypol using Annexin-V FITC kit and FACS analysis

HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were seeded at $1x10^5$ cells per well (in a six well plate) and incubated for three days at 37ºC in a 5% CO_2 humidified atmosphere. On day three, cells were treated with Etoposide alone (10μM – 0.625μM using a 2 fold dilution factor), DCA alone (250mM), Gossypol alone (5mM) and Etoposide (10μM – 0.625 μM) in combination with DCA (250mM) or Gossypol (10mM) for 24 hours and 48 hours. Apoptosis was detected using the Annexin-V-FLUOS staining kit (ROCHE, UK). Further experiments were conducted with DCA at 50mM and 100mM with exposure times of 24 hours and 48 hours and apoptosis was detected using the Annexin-V-FLUOS staining kit.

Following drug treatment, detached cells were harvested by collecting the medium and attached cells were harvested by trypsinisation as described previously. Detached and adherent cells were pooled and all cells were spun down for 3minutes at 800*g*. The supernatant was discarded, and this step was repeated again with 5ml of PBS only. To each sample, equal parts (2 μl) of Annexin V-Fluos labelling reagent (solution A) and Propidium Iodide solution (solution B) was added in a total volume of 100μl (incubation buffer) and incubated for 10min at room temperature. Each sample was placed in an individual FACS analysis tube and a further 400μl of incubation buffer was added to each sample and mixed. The samples were analysed by FACS analysis (BD Biosciences (Oxford UK)). All experiments were carried out in triplicates.

FACS analysis generated raw data which was analysed using WIN-MDI-2.9 provided by Bio-SoftNet Research Tools (http://www.mybiosoftware.com). The

data was presented as a DotPlot graph (figure 2.4). The data was gated to see the four different populations present in the sample and four areas were selected as presented below:



Top Right = Necrotic/Late apoptotic cells

Bottom Right = Apoptotic cells

Top Left = Mechanically damaged cells

Bottom Left = Viable cells

Figure 2.4: A schematic diagram of a DotPlot graph which represents data obtained from FACS analysis after staining with Annexin V displaying different population of cells having undergone different stages of apoptosis after drug exposure. 1 = **Top Left** = Mechanically damaged cells, 2 = **Top Right** = Necrotic/Late apoptotic cells, 3 = **Bottom Left** = Viable cells and 4 = **Bottom Right** = Apoptotic cells represent the different populations of cells detected.

Three populations of cells were observed: (1) viable cells: Annexin V-FITC negative and PI negative; (2) apoptotic cells: Annexin V-FITC positive and PI negative; (3) late apoptotic cells/necrotic cells: Annexin V-FITC positive and PI positive. Early apoptotic cells are stained by Annexin V but not PI because plasma membranes are intact while externalizing Annexin V. Late apoptotic and necrotic cells are stained by both PI and Annexin V as late apoptotic cells

because cells have ruptured membranes. Ruptured cells will stain double positive as the PI can enter the cell and Annexin V can stain exposed phosphatidylserine within the membrane.

Influence of Glutamine on the growth of cancer cell lines

In addition to the isogenic human colon colorectal carcinoma cell lines HCT116 $p53^{+/+}$ (wild type) and HCT116 $p53^{-/-}$ (null type) cell lines described previously, additional cell lines were used. These included MDA-MB-231 (human breast cancer cell line, ATCC HTB-26), MDA-MB-453 human (breast cancer cell line, ATCC HTB-131), MCF7 (human breast cancer cell line, ATCC HTB-22), T-47D (human breast cancer cell line, ATCC HTB-133), and A549 (human lung cancer cell line, ATCC CLL-185), all of which were obtained from the American tissue culture collection (ATCC). In addition, TK10 human kidney renal Cell Carcinoma were also used but these cells were obtained from the DCTD Tumour Repository (http://dctd.cancer.gov). All cell lines were cultured in RPMI 1640 culture medium, supplemented with 10% foetal bovine serum and 1% sodium pyruvate (1mM) and L-glutamine (2mM).

To determine the effect of glutamine deprivation on cell growth, complete medium (500ml without any glutamine supplement) was treated with glutaminase (Glutaminase from Escherichia Coli - 1 unit, Sigma Aldrich, UK) at room temperature for 1 hour followed by 1 hour incubation at 60^{o}C to inactivate the glutaminase enzyme. Glutaminase was added to eliminate any traces of glutamine in the RPMI 1640 and in the serum. Media depleted of glutamine was supplemented with defined concentrations of glutamine ranging from 2mM-0.0039mM using a 2 fold dilution. For each cell line, $5x10^4$ cells were added to

T25 flasks containing 10 ml of medium supplemented with different concentrations of glutamine. Flasks were incubated at 37˚C in a 5% CO_2 humidified atmosphere for 4 days followed by trypsinisation and cell counting for each individual cell line. Visible changes in cellular morphology were also recorded at this stage.

Rescue of cells following glutamine depletion studies

For each cell line (HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, MDA-MB-231, MDA-MB-453, MCF7, T-47D, A549 and TK10) a $1x10^6$ cells/ml cell suspension was made in a final volume of 10ml. Cells were seeded into a T25 flask at a density of $5x10^4$ cells. A total of 16 T25 flasks were seeded per cell line and incubated at 37˚C in a 5% CO_2 humidified atmosphere. For each day (days 1-8) T25 flasks were paired up, the first flask was used for the cell count (as mentioned in chapter 1 method and materials) to determine the effect of glutamine depletion and the second flask had the RPMI 1640 without glutamine removed and replaced with complete RPMI 1640 (containing glutamine) to determine if the cells recovered. The second flask was incubated for a further 4 days at 37˚C followed by a cell count.

Results

The response to cells following a continuous exposure to Gossypol

The response of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells following continuous exposure to gossypol is presented in figure 2.5. Both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines were equally sensitive to gossypol with IC_{50} values 2.56 ± 0.22mM and 2.72 ± 0.29mM respectively. Response to gossypol is therefore independent of the p53 status.

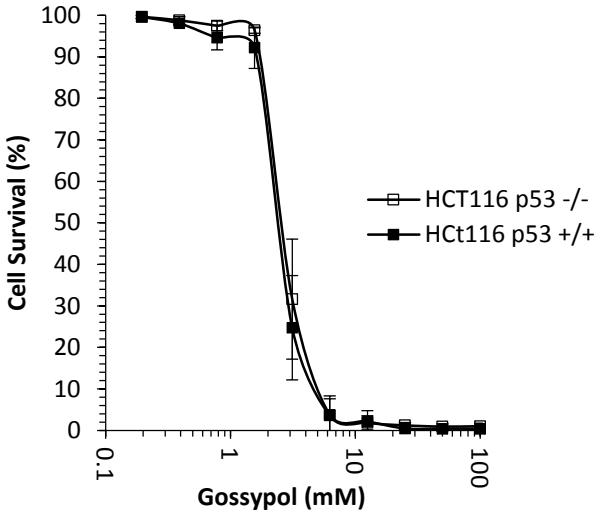


Figure 2.5: Dose response curve following the continuous exposure of HCT116 $p53^{+/+}$ & HCT116 $p53^{-/-}$ cells to Gossypol. Each data point represents the mean ± SD for three independent experiments.

Response of cells following continuous and short term (1 hour) exposures to DCA

The response of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells following continuous exposure to DCA in either RPMI 1640 or DMEM media is presented in figure

2.6. The response of HCT 116 cells in RPMI 1640 (panel A) to DCA was marginally higher (but not statistically significant, P>0.05) in HCT116 $p53^{-/-}$ cells compared to HCT116 $p53^{+/+}$ with IC_{50} values of 16.53 ± 2.95mM and 13.92 ± 2.74mM, respectively. The response of HCT116 cell lines maintained in DMEM culture medium (panel B) differed significantly ($P < 0.05$) from those obtained when cells were cultured in RPMI 1640. IC_{50} values for HCT116$p53^{-/-}$ and HCT116$p53^{+/+}$ were 19.65 ± 0.42mM and 17.03 ± 2.65mM, respectively.

The response of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells cultured in complete RPMI 1640 following 1 hour exposure to DCA is also presented in figure 2.5 (panel A). The IC_{50} value for cell lines HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ are 248.20 ± 15.30mM and 263.97 ± 17.08mM, respectively. In DMEM however, no IC_{50} values were obtained following a 1 hour exposure (figure 2.5 panel B). Both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines did not respond to DCA in DMEM with IC_{50} values of greater than 500mM.

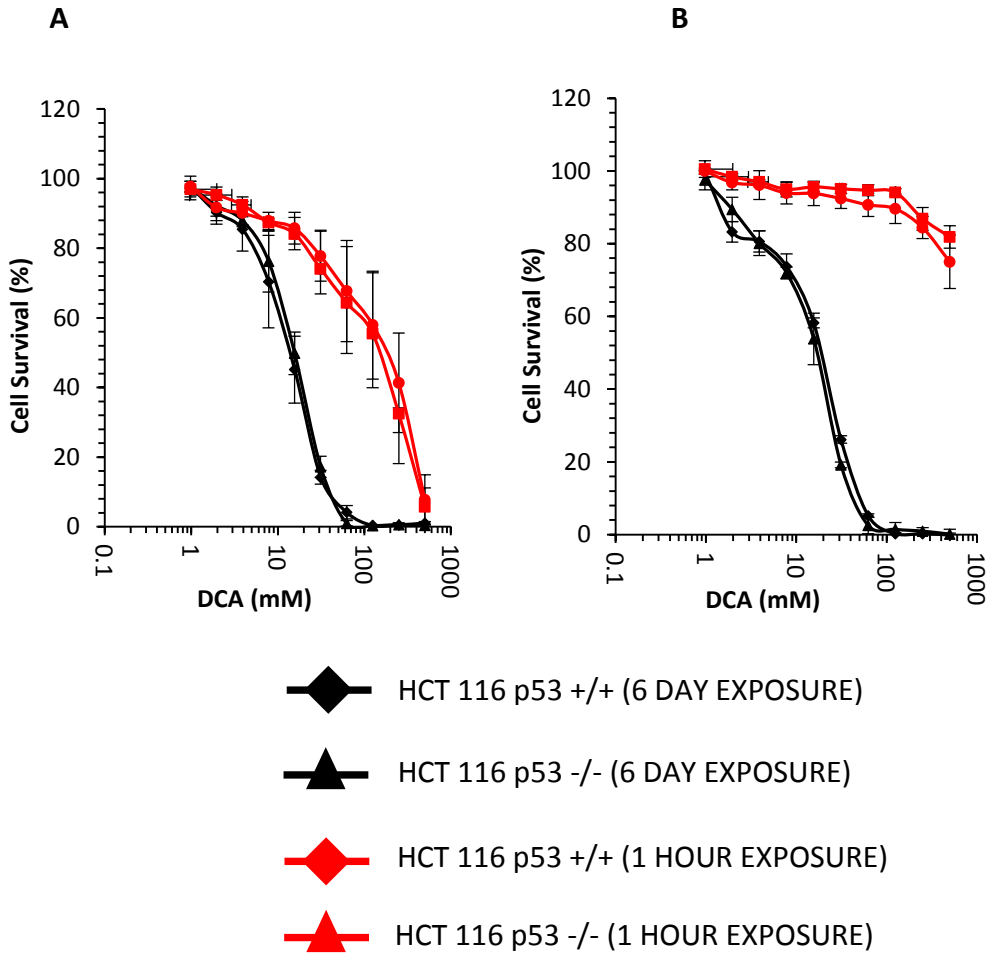


Figure 2.6: Dose response curves for continuous exposure and 1 hour exposure to DCA in HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cell lines. Cells were maintained in complete RPMI1640 (panel A) or DMEM culture medium (panel B). Each value represents the mean ± standard deviation for three independent experiments.

The response of HCT116 cells to TKIs and Bortezomib

The response of cells from cell lines HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ following continuous exposure to the individual TKIs and Bortezomib is presented in figure 2.7 and the IC_{50} values are reported in table 2. Both cell lines HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ were sensitive to all the drugs and the IC_{50} values

demonstrated that cytotoxic activity was independent of the p53 status in all cases. Bortezomib was the most potent as it displayed IC_{50} values in nM concentrations whereas the TKIs typically displayed IC_{50} values in µM concentrations.

The following data is presented in graphs, which displays the dose response curves for each individual TKI in response to HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines:

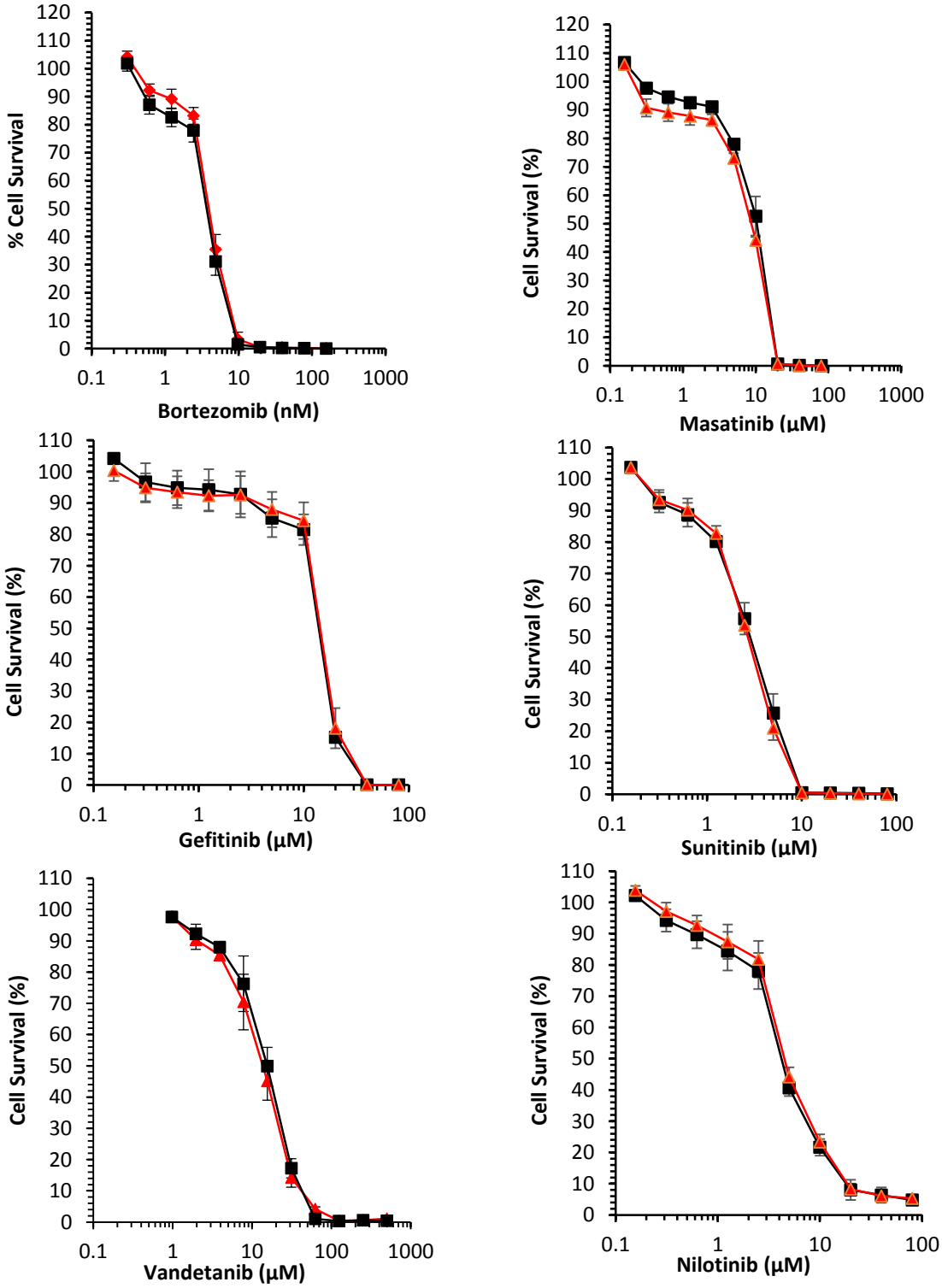


Figure 2.7: Dose response curves after exposure to TKIS's and Bortezomib against HCT116 p53 $^{+/+}$ (black lines) and HCT116 p53 $^{-/-}$ (red lines) cell lines. The exposure time for the drug was constant for 166 hours and each data point represents the mean ± SD for three independent experiments.

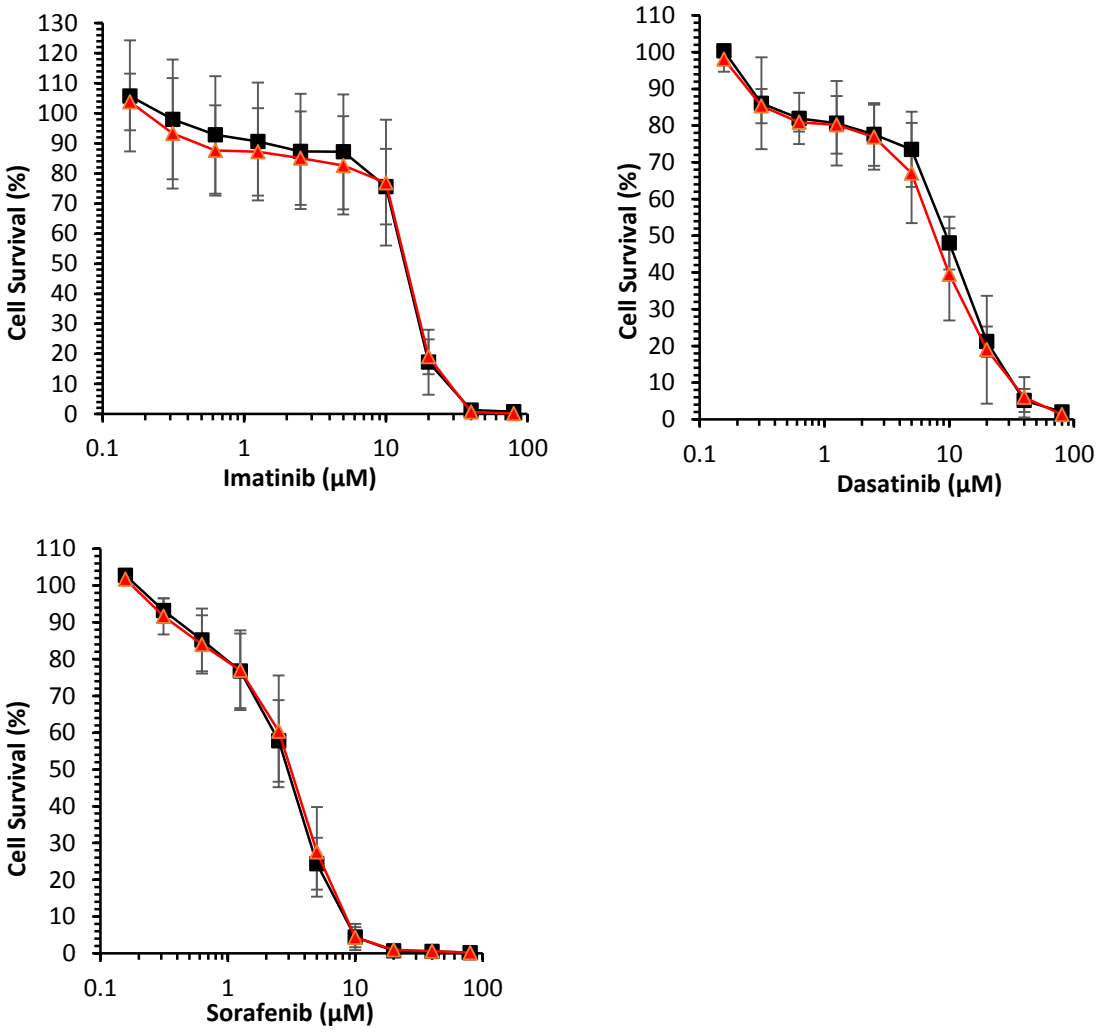


Figure 2.7: Dose response curves after exposure to TKIS's and Bortezomib against HCT116 p53 $^{+/+}$ (black lines) and HCT116 p53 $^{-/-}$ (red lines) cell lines. The exposure time for the drug was constant for 166 hours and each data point represents the mean ± SD for three independent experiments.

DRUG	IC_{50} ± SD (μM) HCT116 p53 $^{+/+}$	HCT116 p53 $^{-/-}$	P Value
BORTEZOMIB	4.16 ± 0.21 (nM)	3.90 ± 0.27 (nM)	0.14
SUNITINIB	2.98 ± 0.45	2.78 ± 0.07	0.26
SORAFENIB	3.03 ± 0.74	3.18 ± 0.99	0.43
NILOTINIB	4.36 ± 0.22	4.60 ±0.23	0.13
VANDETINIB	9.13 ±2.69	10.68 ± 2.63	0.26
DASATINIB	10.13 ± 3.29	9.11 ± 6.94	0.42
MASITINIB	10.54 ± 1.37	9.00 ± 0.17	0.09
IMATINIB	14.33 ± 1.88	13.96 ± 2.31	0.42
GEFITINIB	14.74 ± 0.87	15.32 ± 1.10	0.26

Table 2: The table presents the IC_{50} values obtained from individual TKI's and Bortezomib following continuous exposure to the HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells. Each value represents the mean ± standard deviation from at least three independent experiments. Statistical analysis was carried out and a P value more than 0.05 indicates no significant differences in response to the two cell lines.

The response of cells to DCA combined with TKIs or Bortezomib

To study the effect of TKIs on the activity of DCA, the IC_{50} value for the TKI and a range of DCA concentrations (0.976mM to 500mM) were used. The control using TKIs only were used to calculate percent survival and dose response curves for DCA alone and DCA plus TKI were drawn on the same axis (figure 2.8).

In HCT116 $p53^{+/+}$ cells, IC_{50} values obtained from combinations of DCA and Masatinib, Bortezomib, Sunitinib, Dasatinib, Vandetanib did not significantly differ from IC_{50} values for DCA alone. In the HCT116 $p53^{-/-}$ cells, IC_{50} values obtained from combinations of DCA plus Sorafenib and Vandetanib did not significantly differ IC_{50} values for DCA alone. In contrast IC_{50} values from combinations of DCA and Imatinib, Gefitinib, Nilotinib and Sorafenib were significantly different from DCA alone in HCT116 $p53^{+/+}$ cells. Similarly, IC_{50} values from combinations of DCA and Imatinib, Gefitinib, Nilotinib, Bortezomib, Sunitinib and Dasatinib were significantly different from DCA alone in HCT116 $p53^{-/-}$ cells. In these cases, the TKI's did potentiate the activity of DCA. Furthermore, Sorafenib plus DCA displayed activity that was dependent of the p53 status with potentiation only seen in the HCT116 $p53^{+/+}$ cell line.

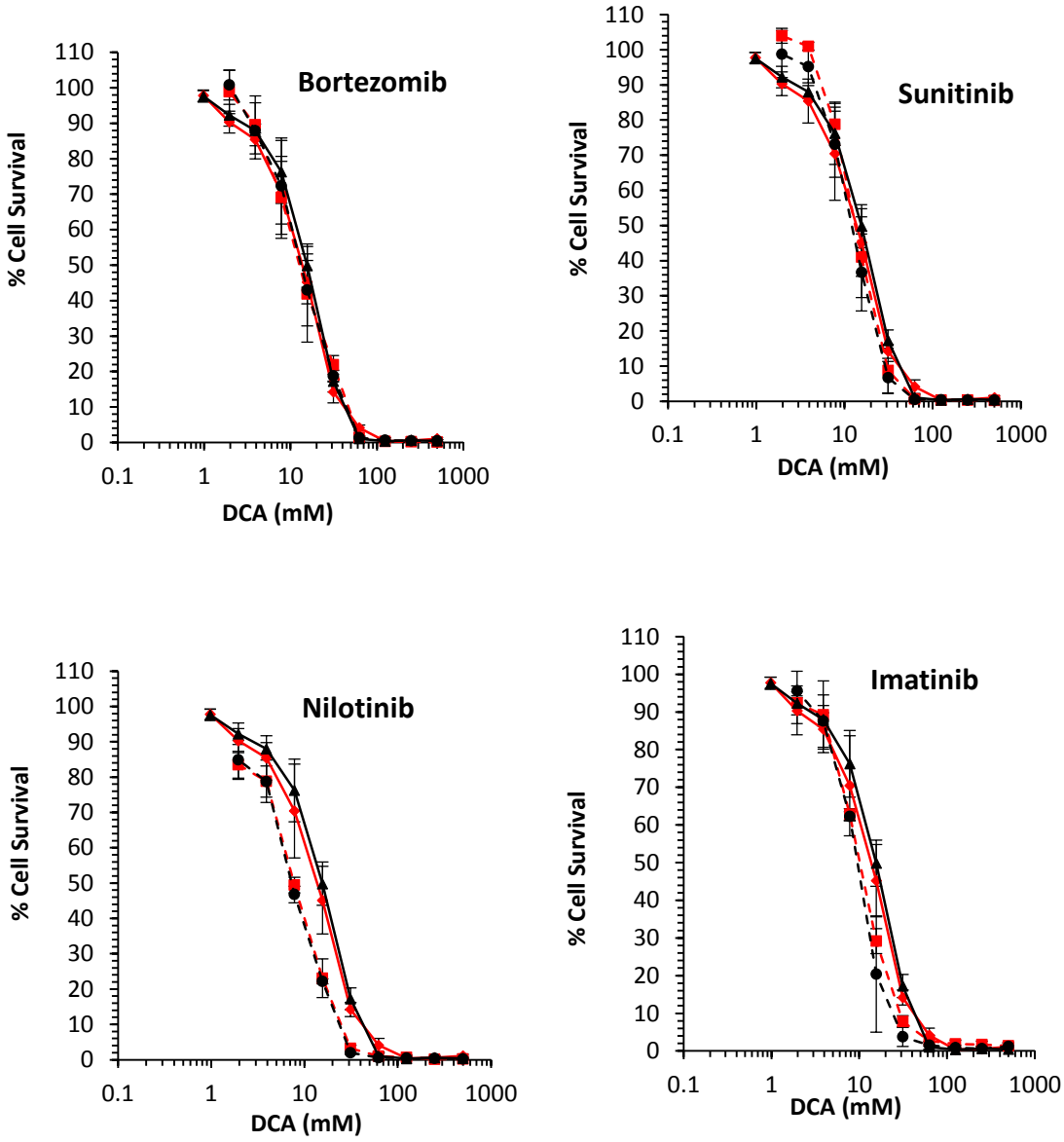


Figure 2.8: Dose response curves measuring the effect of DCA alone and in combination with TKIs. The red symbols and lines represent data for HCT116 $p53^{+/+}$ and the black symbols and lines representing HCT116 $p53^{-/-}$ cells. The red diamond and black square represent cells treated with DCA only. The red square and black circle represent cells treated with combinations of TKI (at the IC_{50} – please refer to table 2) and DCA (various doses). Each data point represents the mean ± SD for three independent experiments.

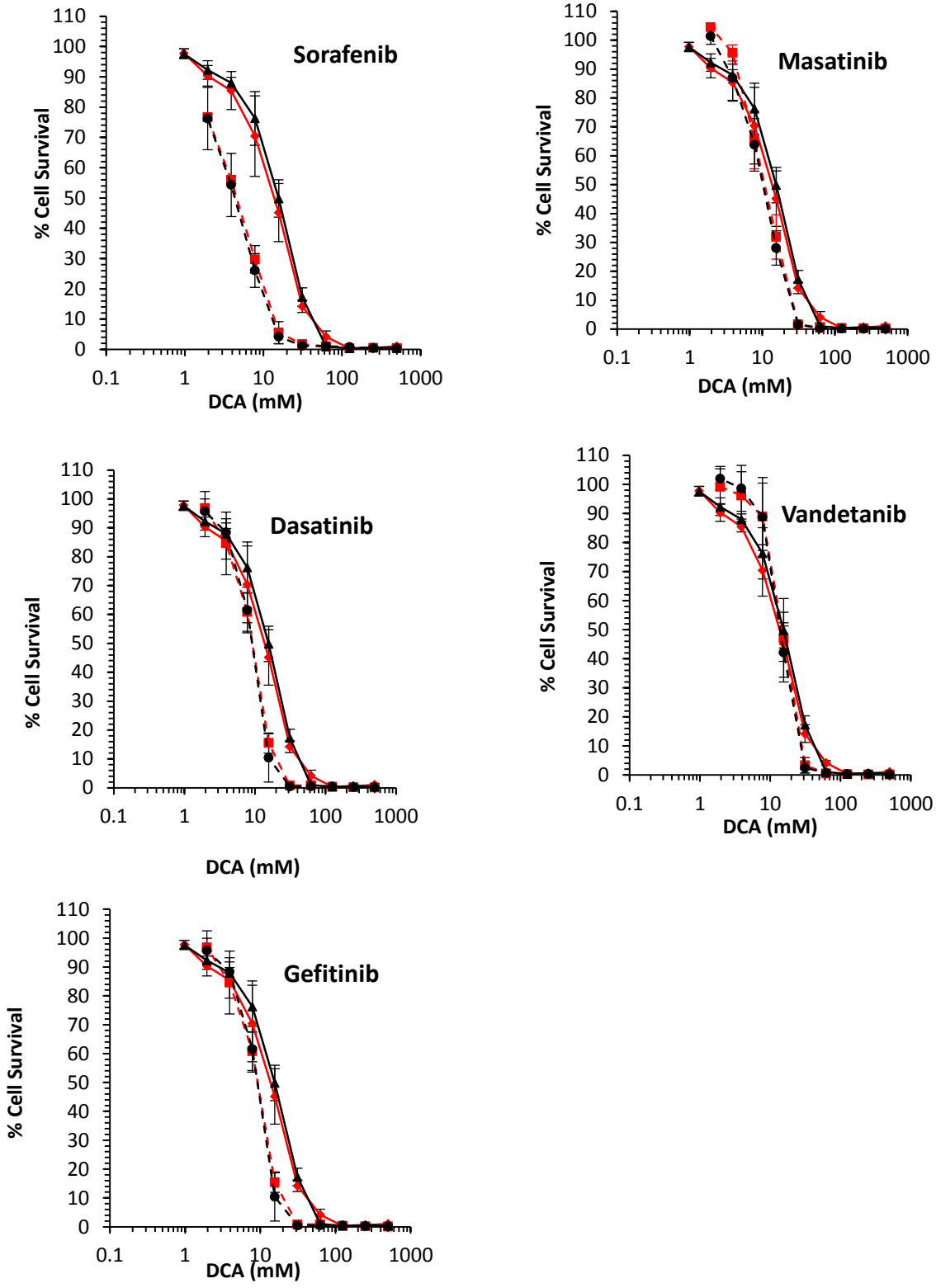


Figure 2.8: (Continued) **Dose response curves measuring the effect of DCA alone and in combination with TKIs.**

	IC_{50} (mM)			
DRUG TREATMENT	**HCT116 p53 $^{+/+}$**	**P Value**	**HCT116 p53 $^{-/-}$**	**P Value**
DCA (ALONE)	13.92 ± 2.74		16.53 ± 2.95	
DCA + SORAFENIB	4.81 ± 0.18	0.005*	16.95 ± 1.15	0.0007*
DCA + NILOTINIB	7.81 ± 0.41	0.008*	7.44 ± 0.23	0.010*
DCA + GEFITINIB	9.60 ± 0.87	0.007*	9.56 ± 1.10	0.010*
DCA + IMATINIB	10.80 ± 1.88	0.0158	10.30 ± 2.30	0.0142*
DCA + MASITINIB	11.16 ± 1.37	0.011	10.55 ± 0.17	0.013*
DCA + BORTEZOMIB	12.42± 0.21	0.045*	13.68± 0.27	0.030*
DCA + SUNITINIB	14.14 ± 2.47	0.16	12.83 ± 2.23	0.075
DCA + DASATINIB	14.74 ± 3.29	0.069	12.62 ± 6.94	0.019*
DCA + VANDETINIB	15.49 ± 2.90	0.37	14.28 ± 1.94	0.15

Table 3: Summary of IC_{50} values for DCA alone and DCA in combination with TKI's. Each value represents the mean ± standard deviation for three independent experiments. Statistical analysis was conducted using a student's t-test comparing IC_{50} values for DCA treated cells against IC_{50} values for DCA plus TKI/Bortezomib treated cells. The asterix indicates statistically significant ($P<0.05$).

Western Blot Analysis

Bradford Assay

The calibration curve was established by preparing serial dilutions of bovine serum albumin (BSA) ranging from 0.03125mg/ml to 1mg/ml. A representative example of linear regression analysis used is presented in figure 2.9 and in this example, the formula y = 0.7586x – 0.0076 was generated. This was used to calculate protein concentrations in the cell extract.

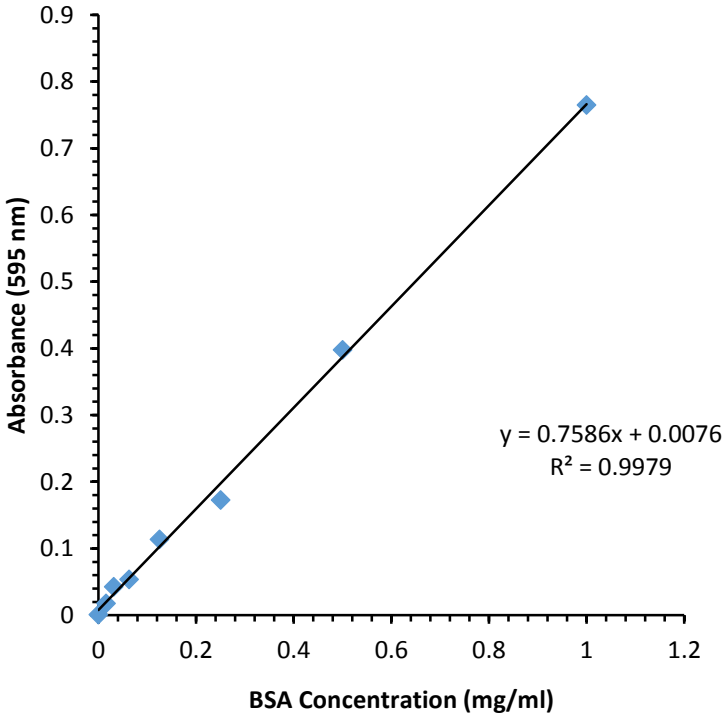


Figure 2.9: Bradford assay calibration curve used to determine the unknown concentration of cell extracts. This is a representative example of a calibration curve used for one experiment. Calibration curves for every experiment were run alongside each unknown set of samples.

Detecting inhibition of PDK1 by DCA

The inhibition of PDK1 by DCA was determined by measuring the phosphorylation of PDH at serine 293. Western blot analysis of PDH, p-PDH and PDK expression in HCT116 cells following a 1 hour exposure to a range of DCA concentrations is presented in figure 2.10. DCA has no effect on the levels of PDH and PDK up to 250mM but at and above 250mM, DCA inhibits the phosphorylation of PDH at serine 293 in both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells. DCA has no effect on the protein levels of PDH or PDK1 indicating that DCA is inhibiting the function of PDK1 at concentrations above 250mM following a one hour drug exposure.

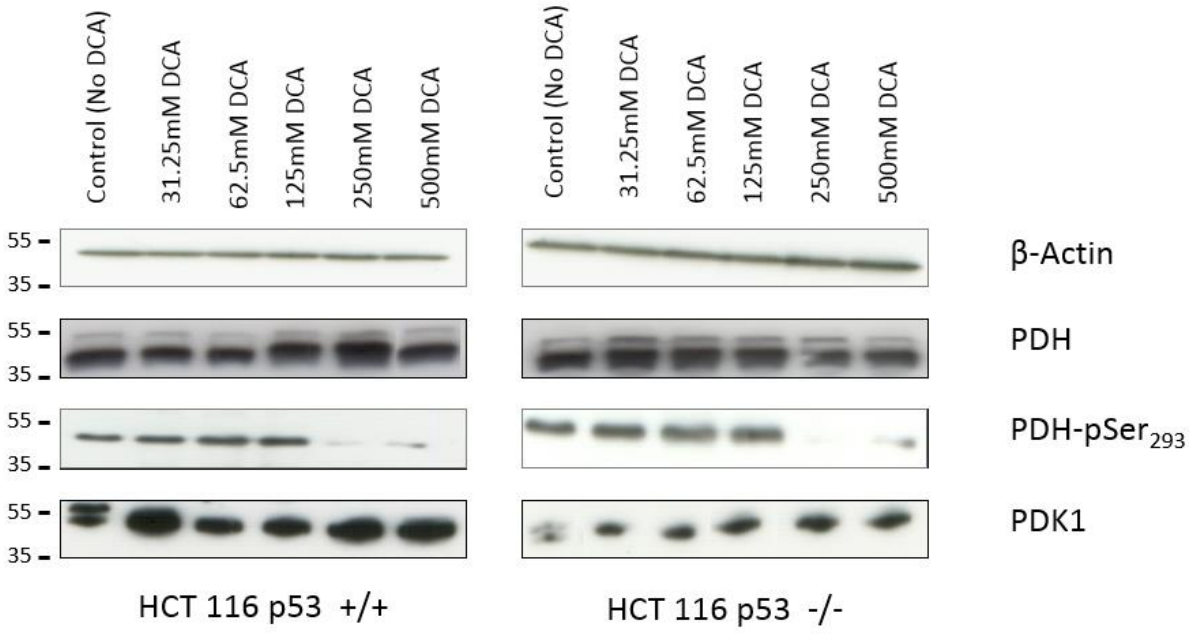


Figure 2.10: Western blot analysis of extracts from HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells treated with a range of DCA concentrations for 1 hour.

Detecting apoptosis induced by therapeutic agents using Annexin-V FITC and FACS analysis

The apoptotic response of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ to DCA, Gossypol and Etoposide alone and in combination (DCA with Etoposide and Gossypol with Etoposide) is presented figure 2.11 part A and B. The FACS analysis results were presented as percentage of the total population of cells that were undergoing apoptosis. The control consists of three conditions, the green bar represents no drug (solvent control – DMSO <0.1%) the red bar represents DCA only (at 250mM) and the blue bar represents Gossypol only (at 10mM). Under the control conditions, levels of apoptosis increased slightly in the presence of DCA alone or gossypol alone but the increase was minor and only approached significance in HCT 116 p53+/+ cells treated with gossypol (figure 3.9A). The remaining data demonstrates that as the concentration of etoposide alone increases, apoptosis increases reaching a maximum of 12.89 ± 03.56mM in HCT116 $p53^{+/+}$ cells and 13.89 ± 0.29mM in HCT 116 $p53^{-/-}$ cells. There is therefore a dose dependent increase in apoptosis following treatment of cells with Etoposide.

In the case of Etoposide combined with DCA (at 250mM), a similar dose dependent increase in apoptosis was observed but at 5 μM and 10μM Etoposide, a significant increase in apoptosis was observed compared to Etoposide alone. Similar results were obtained when Gossypol (at 10mM) was used in combination with Etoposide. These results demonstrate that combining Etoposide with either DCA or Gossypol enhances the activity of Etoposide in terms of inducing a greater apoptotic response (figure 2.11).

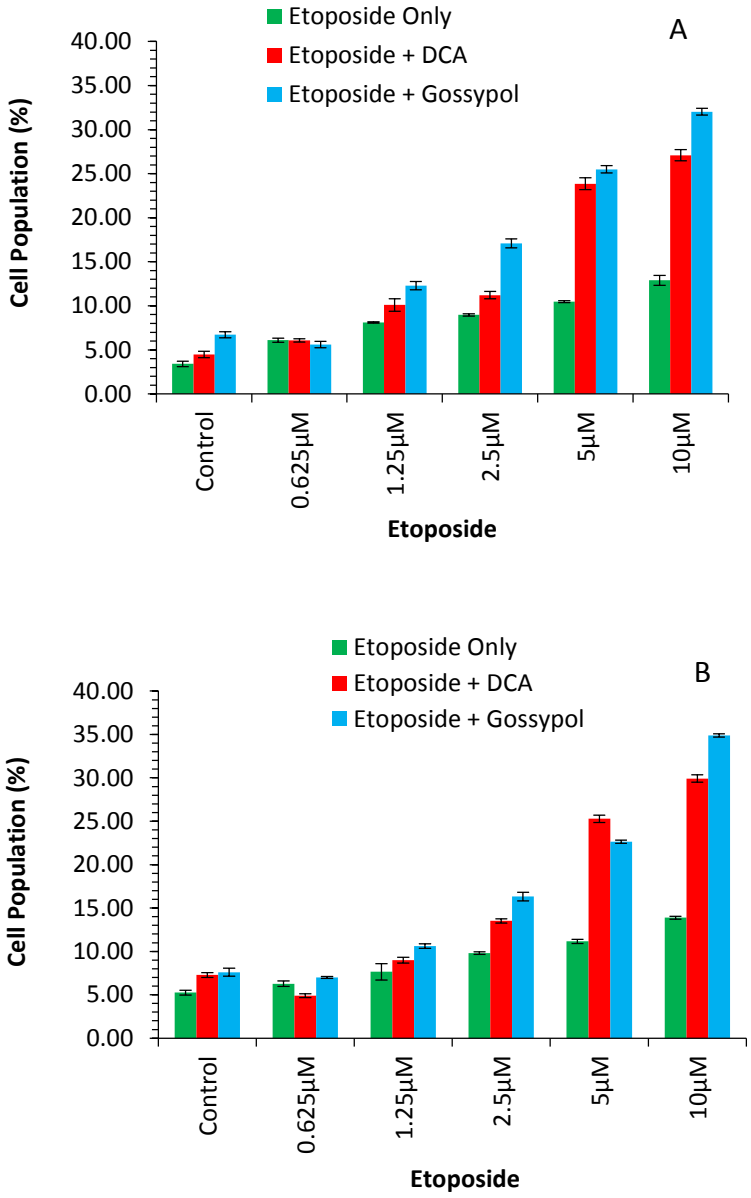


Figure 2.11: Induction of apoptosis following a 24 hour exposure to etoposide alone or etoposide in combination with DCA (250mM) or Gossypol (10mM). Panel A represents the response of HCT116 $p53^{+/+}$ cells and panel B represents the response of HCT116 $p53^{-/-}$ cells. The control values represent drug vehicle alone (green bar), DCA alone (red bar) and Gossypol alone (blue bar). Each value represents the mean ± standard deviation for three independent experiments.

Induction of apoptosis in HCT116 cells following exposure to DCA alone

The induction of apoptosis in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells following exposure to DCA is presented in figure 2.12 panels A-D. The data demonstrates that DCA induces apoptosis in both HCT116 cell lines in both a time dependent and p53 independent manner. Apoptosis was not readily detectable after 24 hour exposure or 48 hour exposure of HCT116 $p53^{+/+}$ cells to DCA (panel A). Similar results were obtained for HCT116 $p53^{-/-}$ cells (panel C). Cells that underwent late apoptosis/necrosis were detected after exposure to DCA and whilst there was no difference in the percentage of cells exhibiting late apoptosis/necrosis following a 24 hour exposure of HCT116 $p53^{+/+}$ cells to DCA (panel B), a significant increase was seen following a 48 hour exposure to DCA (panel B). The level of induction of apoptosis/necrosis was similar for both doses of DCA tested. In HCT116 $p53^{-/-}$ cells (panel D), there was some evidence of a dose dependent increase in late apoptosis/necrosis following a 24 hour exposure to DCA. Following a 48 hour exposure to DCA however, significant induction of late apoptosis/necrosis was observed in HCT116 $p53^{-/-}$ cells (panel D).

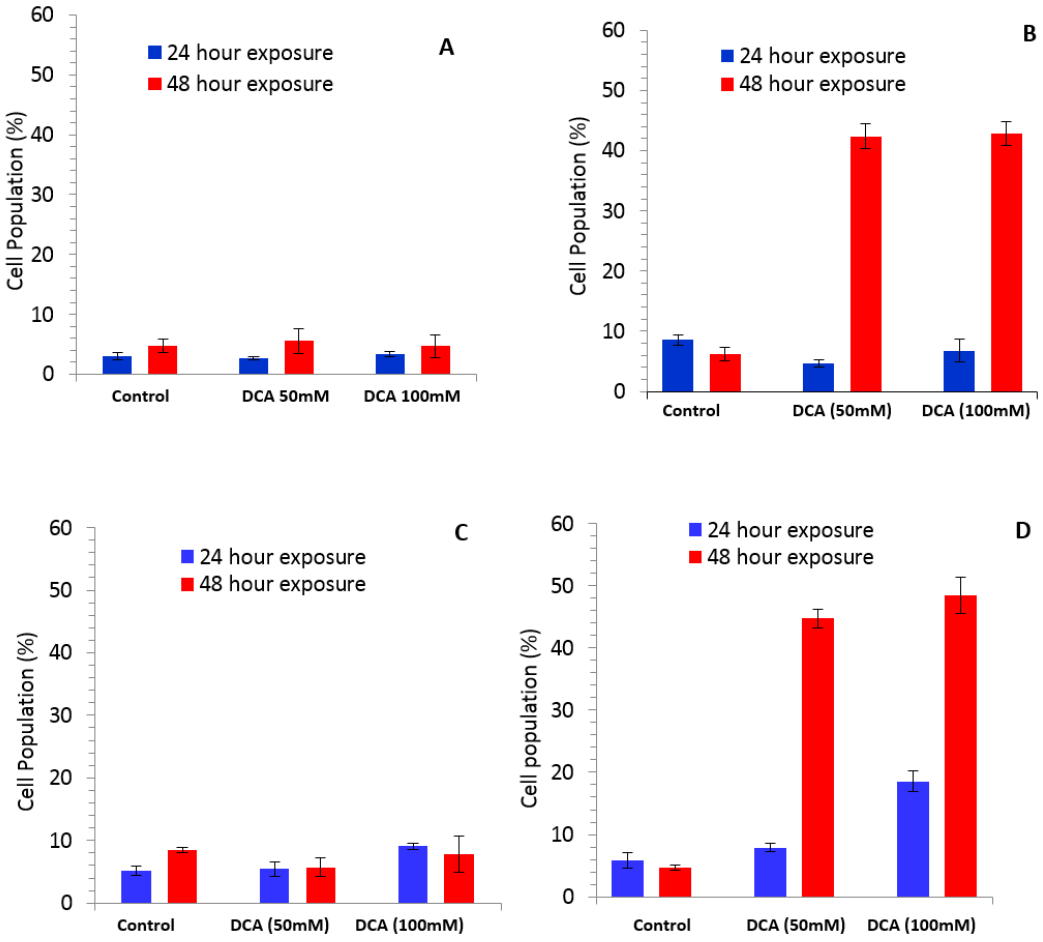


Figure 2.12: Induction of apoptosis is HCT 116 cells following exposure to DCA for 24 or 48 hours. Bar chart A presents the data for HCT116 $p53^{+/+}$ cells following 24 hour and 48 hour exposure to DCA Bar Chart B presents the data for cells undergoing late apoptosis and necrosis following 24 hour and 48 hour exposure from DCA to cells from HCT116 $p53^{+/+}$ cell line. Bar chart C presents the results for HCT116 $p53^{-/-}$ cells following 24 hour and 48 hour exposure to DCA. Bar Chart D presents the data for cells undergoing late apoptosis and necrosis following 24 hour and 48 hour exposure to DCA. Each value present the mean $\pm$ SD for three independent experiments.

Influence of glutamine on the growth of cell lines *in vitro*

The purpose of these experiments was to measure the effect of glutamine on the growth of a panel of cell lines HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, MDA-MB-231, MDA-MB-453, MCF7, T-47D, A549 and TK10. The objective was to determine how much glutamine has to be removed from the media in order to halt cell growth and what happens to cells when glutamine is added to media following a period of glutamine depletion.

The influence of glutamine depletion on the growth of cancer cells *in vitro*

The effect different concentrations of glutamine have on the growth of a panel of cell lines is presented in figure 2.13. In all cases, the media was depleted of glutamine by treatment with glutaminase and the media was subsequently supplemented with defined concentrations of L-glutamine. After 4 days incubation in media, cells were trypsinised and counted. Cell growth was reduced in a glutamine dependent manner in all cases compare to controls (2mM Glutamine). The initial seeding density was 1 x 10^5 cells per flask and with the exception of A549 cells, the cell number in zero glutamine was below the initial seeding density indicating induction of cell death. Even in the presence of low levels of glutamine (3.9μM), cell density was comparable to the initial seeding density in all cases except A549 cells. A549 cells were the only cells tested that were able to grow in the absence of glutamine albeit at a reduced rate compared to controls.

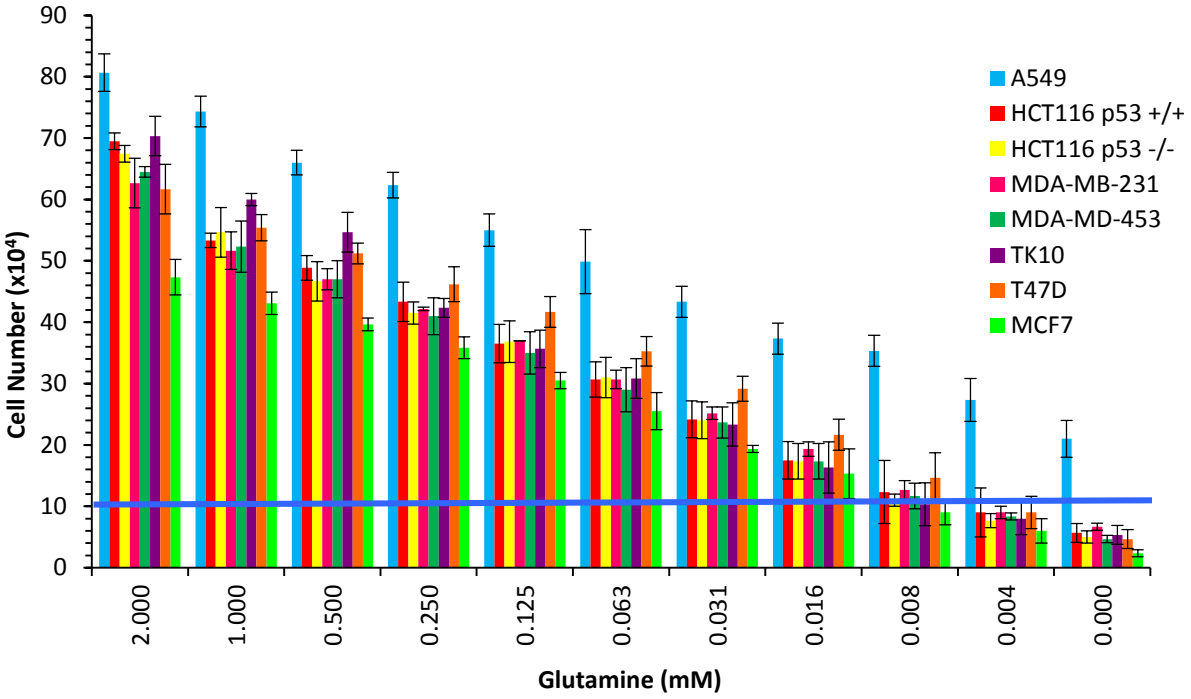


Figure 2.13: A graph presenting a growth curve after exposure to varying concentrations of glutamine against a panel of cell lines. The graphs display the number of cells via cell count (taken 4 days after seeding) against the glutamine concentration. The bar graphs present individual means ± SD for three independent experiments. The initial number of cells seeded was 1 x 10^5 per flask and the solid blue line represents the starting number of cells.

Growth curves in glutamine rich and glutamine depleted media

Cells were seeded at 1 x10^5 cells/flask in glutamine-rich (untreated) media and glutamine-depleted (glutaminase treated) media and incubated at 37ºC for up to 8 days. Every day, cells were trypsinised and cell number determined using the haemocytometer. In addition, changes in cell morphology occurring throughout the experiment were observed and recorded following visual inspection. The

results are presented in figures 2.14, in all cases, the number of cells attached to the base of culture flasks decreased significantly within 24 hours of seeding in glutamine-depleted media. Under these conditions, cell adherence was poor with large numbers of cells remaining in suspension. In contrast to the control cultures in glutamine rich medium, cell growth was significantly reduced in glutamine-depleted media. In all cases, cell growth, did occur up to day 4 or 5 but after this, cell growth decreased with clearly visible signs of cell death observed under the microscope.

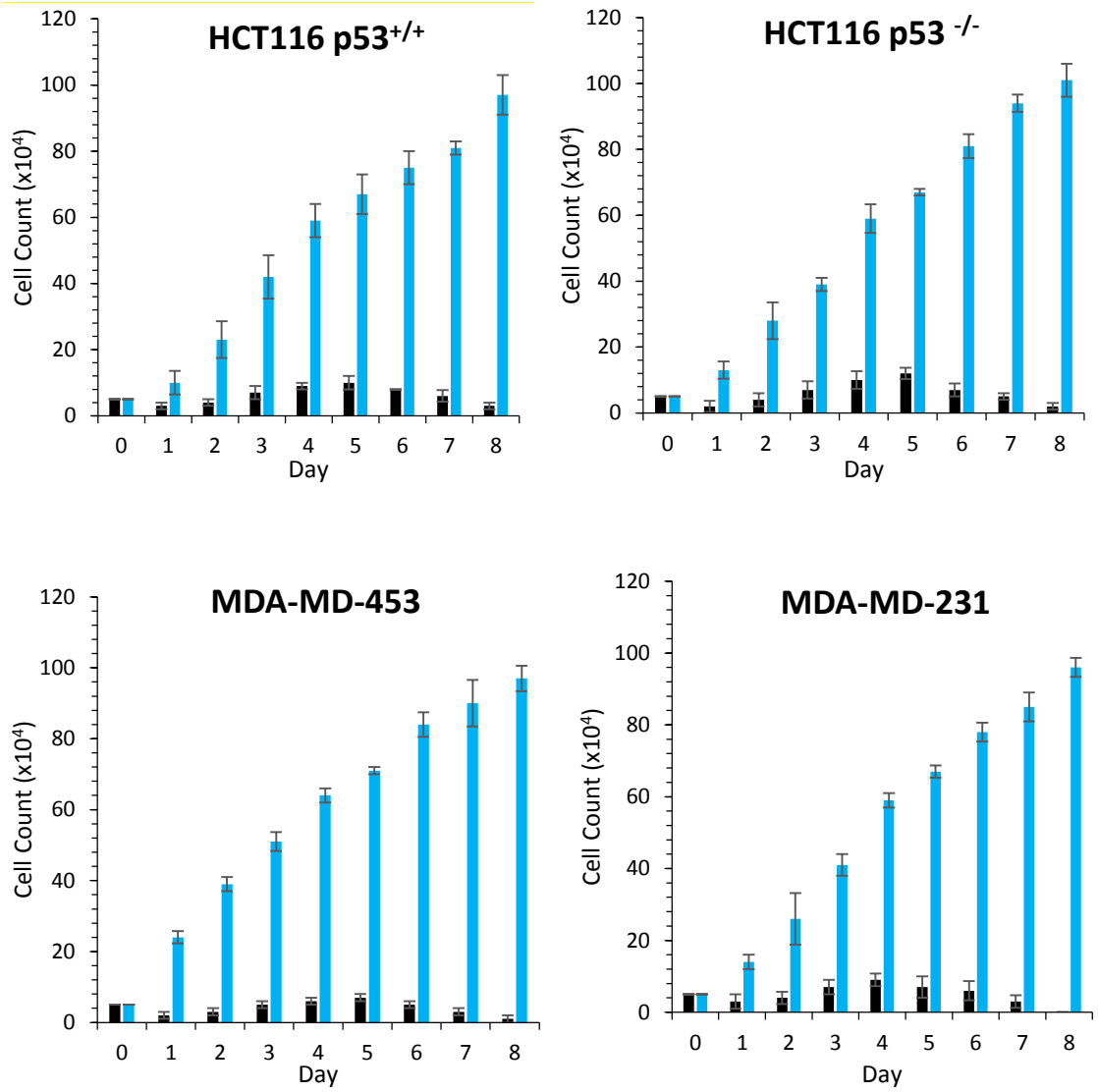


Figure 2.14: Growth curves measuring the effect of glutamine on a panel of cell lines. The graphs represent growth of cells in the presence and absence of glutaminase in the culture medium by cell count over a period of 8 days, day zero is when the cells were seeded. The bars to the left represent glutamine-depleted (black) and bars to the right represent glutamine rich media (blue) respectively. Each data point represents the mean ± SD for three independent experiments.

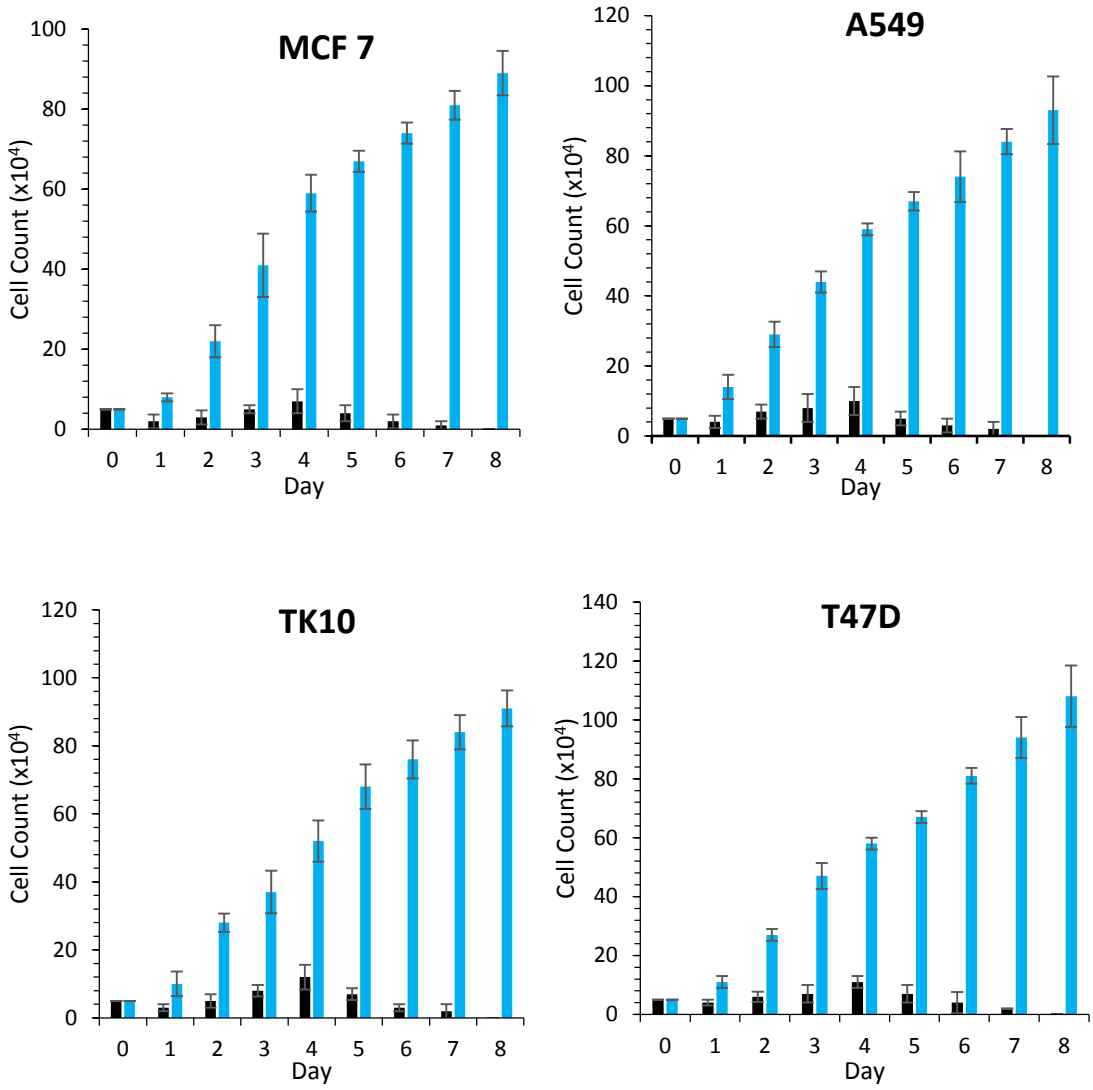


Figure 2.14: (Continued) **Growth curves measuring the effect of glutamine on a panel of cell lines.**

Rescue of cell growth following glutamine depletion

Cells were seeded at 1 x 10^5 cells per flask and incubated for up to 8 days in glutamine-deprived media as described above. Each day, the glutamine-deprived media was removed and replaced with glutamine-rich media and cells were allowed to recover in this media for up to 4 days following which a cell count was performed. The results are presented in figure 2.15. Following a 1 day incubation in glutamine-deprived media, cells rapidly recovered and good cell growth was observed. This was apparent for all cell lines tested. At the other end of the spectrum, no recovery was observed following an 8 day incubation of cells in glutamine-deprived media. At time intervals between these two extremes, the magnitude and timing of cell line recovery varied depending upon the cell line (figure 2.13). These results demonstrate that glutamine levels need to be reduced to low levels for long periods of time in order to induce a significant cytotoxic effect. Furthermore, replenishment of glutamine levels following a short period of glutamine depletion will lead to rapid repopulation of the cell population. A459 cells showed little effect to glutamine deprivation, although when the glutamine from the medium was removed A549 cells maintained proliferation but at a slower rate. Nevertheless, A549 cells continued to survive and proliferate unlike the other cell line (figure 2.13).

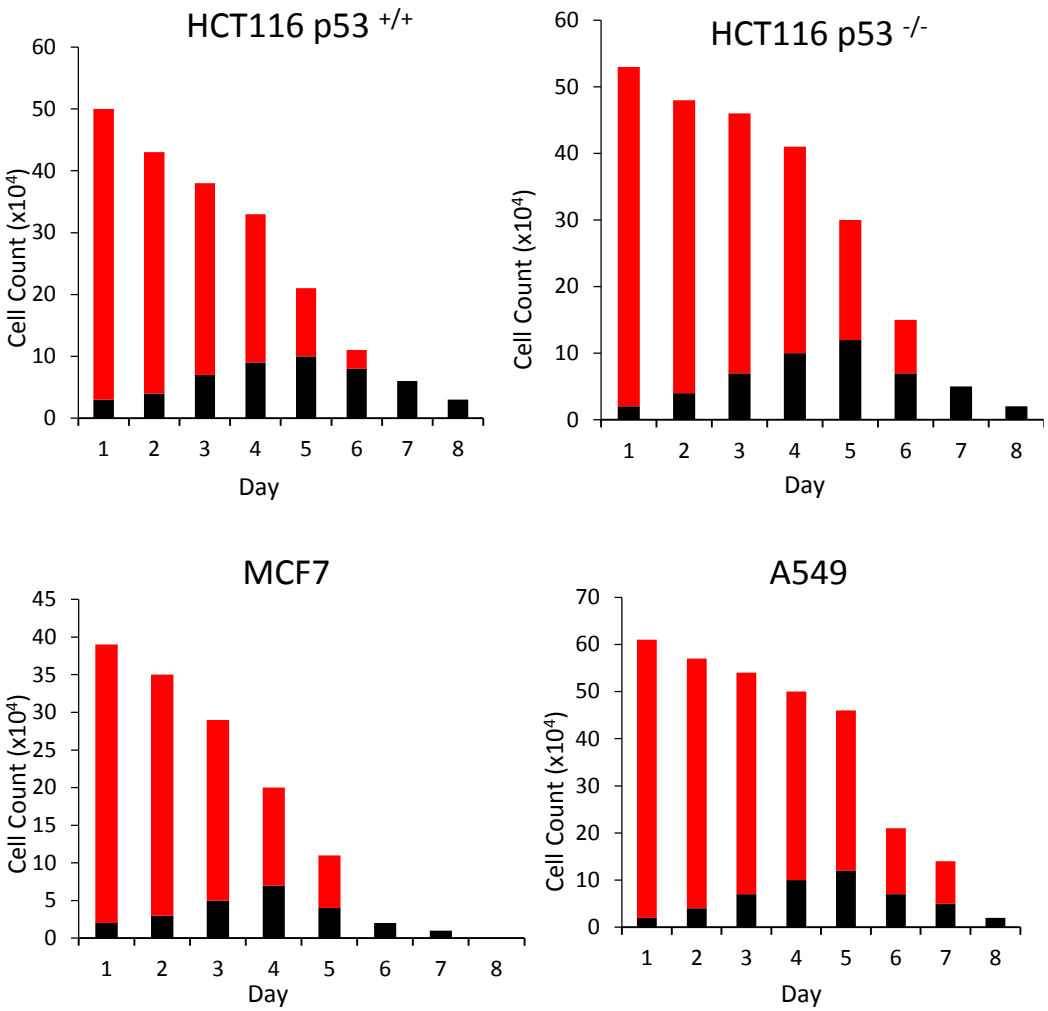


Figure 2.15: Recovery of cell growth following a period of glutamine depletion. Cells were incubated in glutamine-deprived conditions (black bars) and cell counts performed over a period of 8 days. At each stage of the growth curve, media was replaced with glutamine-rich media (red bars) and cells were allowed to recover for a further 4 days before cell number was determined.

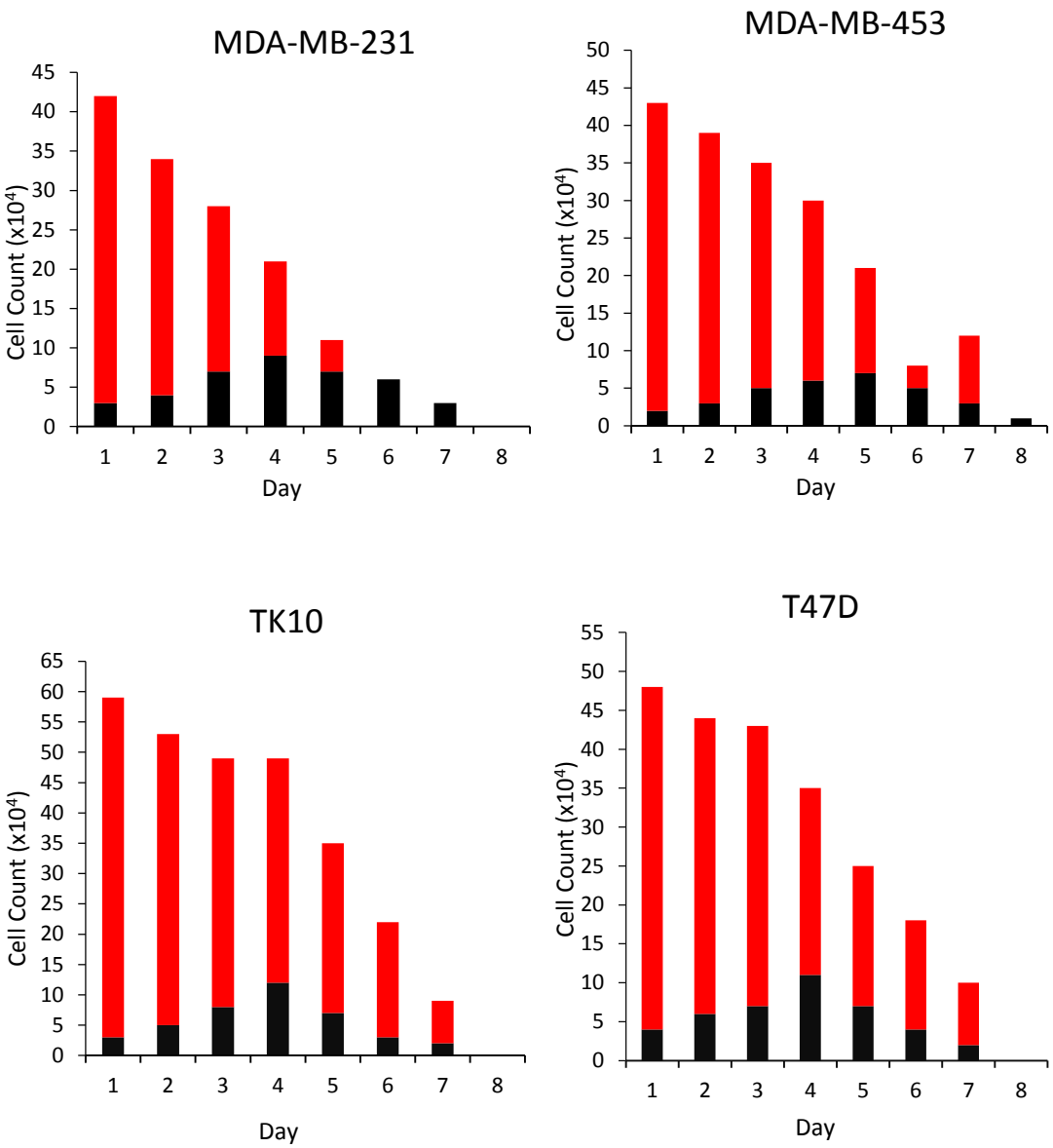


Figure 2.15: (Continued) **Recovery of cell growth following a period of glutamine depletion.** Cells were incubated in glutamine-deprived conditions (black bars) and cell counts performed over a period of 8 days.

Discussion

Whilst considerable progress has been made in reducing mortality in some types of cancer, mortality rates for other cancers remain high and there is a pressing need to develop novel therapeutic approaches. Understanding the biology of the disease and the pharmacology of drugs used to treat cancer is driving forward the development of novel anti-cancer drugs and novel strategies to discover new drugs and make existing drugs work better.

The primary objective for this chapter was to see whether or not the Warburg effect can be pharmacologically manipulated to enhance the activity of targeted anti-cancer drugs. Specifically, inhibitors of LDH-A and PDK1 were evaluated in a panel of cell lines and combination studies *in vitro* conducted using a series of clinically approved tyrosine kinase inhibitors and Bortezomib. Glutamine addiction is also a common feature of the Warburg effect and a further objective was to determine what effect glutamine depletion strategies have on the viability and response of cells *in vitro*. The experimental studies investigated the 'depth' of glutamine depletion required to induce cellular responses and whether or not the effects of glutamine depletion are reversible once glutamine levels return to normal. The key results are discussed here in the context of the primary objective stated above.

With regards to the cytotoxic activity of Gossypol and DCA alone, both proved to be cytotoxic to cells but only at doses that are typically much higher than cytotoxic drugs. To begin with Gossypol, a LDH A inhibitor was found to possess cytotoxicity activity against both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines in a dose dependent manner and independent of the p53 status. Ponzak *et al*

found that Gossypol is cytotoxic and does in fact induce cell death inhibiting cancer cell growth (Van Poznak et al., 2001). DCA is a known PDK1 inhibitor also possesses cytotoxicity against both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines in a dose dependent manner and independent of the p53 status. Michelakis *et al* (2008) found that DCA is cytotoxic to the cells which results in inhibition of cancer cell growth and proliferation (Michelakis et al., 2008).

Working on HCT116 cells which are p53 wild type ($^{+/+}$) or null ($^{-/-}$) is an important aspect of the study as the p53 plays a key role in apoptosis pathway. The p53 status is also important as p53 is implicated in many metabolic pathways and therefore an important aspect in research. The IC_{50} values for Gossypol and DCA clearly indicate they both induce cell death or inhibition of cell growth and are independent of the p53 status. The p53 tumour suppressor gene is mutated in over 50% of human tumours and plays an important role in the response to genotoxic stress and hypoxia (Vogelstein et al., 2000). p53 responds to upstream signals by activating transcription of genes important for cell cycle arrest, DNA repair, and apoptosis (Kaeser et al., 2004). It is therefore somewhat surprising that no differences in IC_{50} occurred following exposure to Gossypol and DCA but the data clearly indicates that response is independent of p53.

To demonstrate that the dose of DCA used caused inhibition of PDK1, HCT116 cell lines were analysed using the Western blot method to detecting specific proteins of interest. These were PDH, phosphorylated-PDH (p-PDH) and PDK1. The Western blots confirmed that at higher doses of DCA (250mM and 500mM) PDK1 was inhibited resulting in a reduction in p-PDH levels consistent with reactivation of PDH. These results are consistent with previously published studies (Cao et al., 2008). Following a 1 hour exposure to DCA, the dose of DCA

required to induce cytotoxic effects (figure 2.6) is directly comparable to the dose required to inhibit PDK1 (250mM). Whether the induction of cytotoxic effects is due to inhibition of PDK only is unclear and further studies would be required to determine this. Nevertheless, these results confirm that DCA alone is cytotoxic to cells *in vitro* (Heshe et al., 2011; Michelakis et al., 2008) and confirm that DCA is inhibiting PDK1 at doses that correlate with cytotoxic activity.

Having demonstrated that Gossypol and DCA are cytotoxic *in vitro*, the next point of investigation was to confirm whether Gossypol and DCA possess the ability to reactivate apoptosis. Using Annexin-V fluorescent staining, exposure of cells to Gossypol or DCA resulted in the reactivation of apoptosis (figure 2.11). These results are consistent with other studies. Volate *et al* and Madhok *et al* reported that Gossypol and DCA can both reactivate apoptosis in cancer cells (Madhok et al., 2010; Volate et al., 2010). In contrast to Gossypol, FACS analysis demonstrated that DCA induced a significant number of necrosis in comparison to apoptosis (figure 2.12). An explanation for this, is in the understanding of the three populations of cells, which were observed in figure 2.4: (1) viable cells: Annexin V-FITC negative and PI negative; (2) apoptotic cells: Annexin V-FITC positive and PI negative; (3) late apoptotic cells/necrotic cells: Annexin V-FITC positive and PI positive. Early apoptotic cells are stained by Annexin V but not PI because plasma membranes are intact while externalizing Annexin V. Late apoptotic and necrotic cells are stained by both PI and Annexin V because cells have ruptured membranes. Ruptured cells will stain double positive as the PI can enter the cell and Annexin V can stain exposed phosphatidylserine within the membrane (Wlodkowic et al., 2011).

As a single agent, DCA has proven to have anti-tumour efficacy in preclinical models (Michelakis et al., 2008) and is in clinical trials. Its principle application may, however, be in combinations of DCA with other drugs. DCA is known to affect a cells ability to undergo apoptosis. DCA is known to potentiate the activity of certain drugs e.g. Temozolomide (Michelakis et al., 2010), Doxorubicin (Heshe et al., 2011) and platinum based drugs (Olszewski et al., 2010) but no studies have assessed whether DCA can influence the activity of TK inhibitors or Bortezomib. RTKs are an integral part of cancer cell progression and contribute to the Warburg effect. To determine whether combinations of DCA and targeted therapeutics results in greater efficacy, this study first of all characterised the response of cell lines to TKIs and Bortezomib as single agents. The results demonstrated that Bortezomib was the most potent compound tested having cytotoxic effects on the cell in nM concentrations whereas, the TKIs were all active in the μM range. TKIs and Bortezomib as single agent drugs were equally cytotoxic to both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines demonstrating that the activity of TKIs and Bortezomib is independent of the p53 status. p53 has been known to influence the response of chemotherapeutic agents leading to resistance (Brown and Wouters, 1999; Fridman and Lowe, 2003). It is well documented that the p53 plays a major role in apoptosis and due to mutations deficient p53 cells gain an advantage, losing the ability to induce apoptosis a hallmark of cancer (Kaufmann and Earnshaw, 2000). Re-establishing apoptosis in cancer cells can lead to inhibition in cell growth and proliferation reducing tumour size (Zhang et al., 2000) therefore the influence of p53 is an important consideration in this research.

TKIs and Bortezomib in combination with DCA reduced the IC_{50} of DCA when compared to DCA alone. HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells displayed differential cytotoxicity to different TKIs and Bortezomib alone, in combination with DCA enhanced potentiation was seen in selective TKIs and Bortezomib. Furthermore, TKIs and Bortezomib improved the efficacy of DCA when combined with some but not all of the TKIs and Bortezomib. Exposure to DCA in the presence of Bortezomib, Sorafenib, Masitinib, Imatinib and Gefitinib to cells from cell line HCT116 $p53^{+/+}$ showed synergy, the IC_{50} values reported showed a decrease in IC_{50} values when compared to DCA alone (Table 3). In respect to the dose response curves a shift to the left was observed suggesting that the presence of the TKIs in combination potentiated the effects of DCA. As for the HCT116 $p53^{-/-}$ cell line, exposure to the TKIs and Bortezomib combined with DCA (except Sorafenib) produced lower IC_{50} values when compared with the IC_{50} value of DCA alone. Bortezomib was the most potent of the drugs screened in this chapter as IC_{50} values obtained were in nM concentrations when exposed alone. The IC_{50} values obtained illustrated that the p53 status of the HCT116 cell lines did not influence the inhibitory effects of DCA and TKIs or Bortezomib in combination except for Sorafenib (figure 2.8 and table 3). Sorafenib displayed IC_{50} values that indicated that the p53 status of the HCT116 may play a role in respect to toxicity. The mechanistic basis for this is not known however and further studies are required to explore the p53 dependent effects observed in this study.

In summary, the results of this study have demonstrated that the combination of TKI's or bortezomib with DCA can significantly influence the activity of DCA. This is the first time a potential link between the Warburg effect and the activity of

drugs targeted at aberrant cell signalling pathways has been demonstrated. Further studies are required to optimise the combination conditions and to understand the mechanistic basis underpinning these results. Nevertheless, they suggest that modulators of cellular metabolism could help existing drugs work better.

Moving on to look at cancer cells addiction to glutamine, the aim was to critically evaluate whether glutamine depletion strategies have potential therapeutic applications. This research addressed many of the key issues relating to the 'depth' of glutamine depletion required to induce cellular responses and whether or not the effects of glutamine depletion are reversible once glutamine levels return to normal.

A variety of human cancer cell lines have shown sensitivity to glutamine starvation, including pancreatic cancer, glioblastoma multiforme, acute myelogenous leukemia, and small cell lung cancer (Wise and Thompson, 2010). Studies have detected an up-regulation of high affinity glutamine transporters in cancer (Fuchs and Bode, 2005). To understand the effects of glutamine on various cell lines this chapter investigated the response of cells to varying concentrations of glutamine over a period of 1-8 days. The data suggested that as the concentration of glutamine is reduced, the rate of proliferation is also reduced suggesting that lack or absence of glutamine does alter the proliferation and growth of cancer cells. These results support the notion that that cancer cells do require glutamine and the presence glutamine is an important driver of cancer cell proliferation. It also supports the notion that depriving cancer cells of

glutamine will impact upon their ability to proliferate and it therefore a potential therapeutic strategy.

Growth curves for cells maintained in glutamine free medium demonstrated that the depletion of glutamine impacts the cells ability to proliferate in comparison to glutamine rich medium. Cells exposed to culture medium with no glutamine resulted in low cell counts and many of the cells dying on progressive days. On the other hand cells maintained with glutamine presented normal growth as the number days increased the cell count for each day increased. Cells demonstrated an addictive behaviour towards glutamine, which was observed in the panel of cell lines. The cells were observed under the microscope, after days 2/3 the cells from HCT116 cell lines displayed a change in cell morphology and further went on to lose adhesion.

Furthermore, the panel of cells lines where starved of glutamine followed by reintroducing glutamine. Lack of glutamine resulted in decreased proliferation and lead to cell death but cells responded by recovering and returned to growth when glutamine was re-introduced. This experimental data further reinforces the fact that cancers cells are indeed addicted to glutamine (Daye and Wellen, 2012). After days 4/5 depending on the cell line, cells maintained without glutamine were more susceptible to cell death and upon re-introduction of glutamine the cells were no longer able to proliferate and sustain growth. Cells reintroduced to glutamine before days 4/5 go on to recover and progressively continue to grow in numbers. Research carried out by Mariia *et al.* and her research team found that normal cells are also dependent on glutamine. They used normal human diploid human lung fibroblasts IMR-90, normal diploid

human foreskin fibroblasts and found that a deficiency in glutamine killed most of the human cells (Yuneva et al., 2007).

In relation to glutamine and its role in cancer this chapter has provided experimental data which is in broad agreement with current literature (Reinert et al., 2006; Yuneva et al., 2007). The fact that cytotoxic effects were only observed after prolonged glutamine depletion does raise some concerns about the effectiveness of this approach as a single agent strategy. If glutamine has to be kept at low levels for such prolonged periods of time, it's unclear what effect this would have on normal cells. Toxicity is therefore likely to limit the effectiveness of glutamine depletion strategies (Yuneva et al., 2007). In addition, glutamine levels have to reduce to very low levels in order to see cytotoxic effects. This is also a concern as it will be difficult to deplete glutamine levels below the concentrations required to kill cells. Nevertheless, glutamine depletion could be valuable when combined with other approaches being developed to target glutamine metabolism and it should be investigated further. (DeBerardinis and Cheng, 2010; Wise and Thompson, 2010).

Future research

There are aspect of this research which can be exploited further, the current research looks at DCA in combination with TKIs yet there is scope for the combination of TKIs with Gossypol. Furthermore, Gossypol and DCA can be exposed to a panel of cell lines to see whether the toxicities are specific to certain cancer type or general to all cancers types.

In terms of combination studies, potential research avenues in the future could be switching the parameters between DCA and TKIs. DCA was the variable drug and the TKIs and Bortezomib were constant in this study. By making the TKIs and Bortezomib the variable drug and DCA as the drug which is kept constant, improved IC_{50} values could be achieved. The experimental data from the combination studies between DCA and TKIs looked promising, it would be beneficial if the cells underwent detection for apoptosis. This would provide data on the mechanism by which the drugs may induce cell death, if in fact the combination of drugs reactivated apoptosis.

Moving on to the experimental data for the glutamine experiments, visual observations from the microscope showed that after a certain number of days either day 4/5 depending on cell line, cells maintained without glutamine were more susceptible to cell death and upon re-introduction of glutamine the cells were no longer able to proliferate, the reason behind these observations are still unclear and will need investigating. In the presence of no glutamine, cells were losing adhesion capability and also the morphology of cancer cells changed an aspect which could not be explained. Therefore investigating these unknowns would help understand the effect of glutamine in cancer cells and why the withdrawal of glutamine has such profound effects on adhesion and physiology. Furthermore glutamine inhibitors such as Kidrolase and Erwinase would be attractive in discovering the effect of glutamine deprivation on cells and the possible benefits to therapeutic outcomes and benefits to patients.

CHAPTER THREE

Introduction

Measuring the activity of Temozolomide analogues against MGMT and MMR deficient cell lines in the absence and presence of PaTrin2

Alkylating drugs are the oldest class of anticancer drugs still commonly used and they play an important role in the treatment of several types of cancers. They are a class of chemotherapy drugs that bind to the DNA and prevent the process of DNA replication by directly damaging the DNA. Alkylation involves the addition of alkyl group to the *N*- and *O*- atoms in DNA bases. Methylation adducts at the *N*- terminal account for up to 80% methylated bases (Kondo et al., 2010). Since cancer cells in general, proliferate faster than healthy cells, cancer cells are more sensitive to DNA damage. There are different classes of alkylating agents, including:

- Nitrogen mustards: such as mechlorethamine, ifosfamide, and Melphalan
- Nitrosoureas: which include streptozocin, carmustine (BCNU), and lomustine
- Alkyl sulfonates: busulfan
- Triazines: dacarbazine (DTIC) and Temozolomide (Temodar)
- Ethylenimines: thiotepa and altretamine (hexamethylmelamine)

However, alkylating agents are also inherently toxic to normal cells, leading to damage in healthy normal cells. The damage is particularly acute in normal cells that divide frequently, such as those in the gastrointestinal tract, bone marrow, testicles and ovaries. By virtue of the fact they cause damage to DNA at sub-

lethal doses, most of the alkylating agents are also known to be carcinogenic (Kondo et al., 2010).

The development of Temozolomide (TMZ) started with Mitozolomide (MTZ) which is an imidazotetrazine. The imidazotetrazine ring is an acid stable precursor and prodrug of highly reactive alkyl diazonium ions that ultimately leads to DNA damage via methylation.

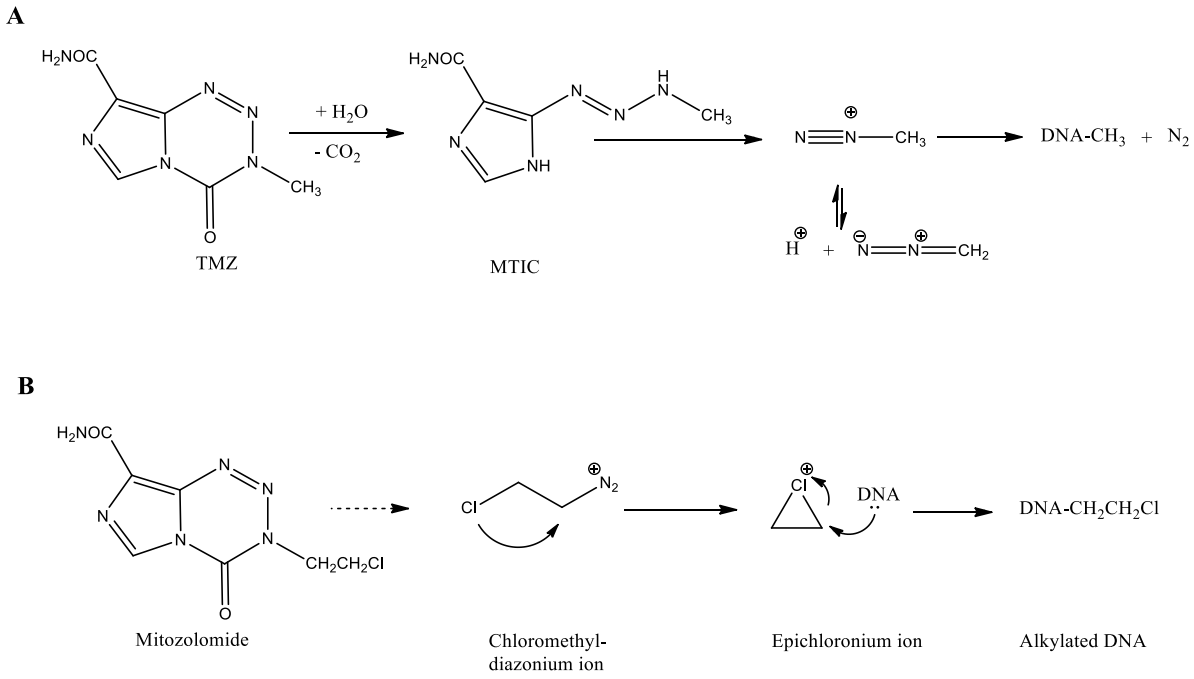


Figure 3.0: Schematic diagram (A) for the mechanism of action of Temozolomide and Mitozolomide (B). Temozolomide is converted in to MTIC which methylates the DNA resulting in DNA nicks. Due to the DNA being damaged the cell induces apoptosis (Ramirez et al., 2013). Mitozolomide is converted in to chloroethyl-diazoniumion which alkylates the DNA resulting in apoptosis (Fang et al., 2012).

MTZ is a prodrug that spontaneously decomposes to a highly reactive DNA cross linking metabolite. MTZ can either bind two DNA molecules together or can bind at two different sites on the same DNA molecule making it an effective anti-cancer drug. MTZ had broad spectrum activity against many tumours and by Fiebig et al and his team identified the potential benefits of MTZ including curing some tumours (Fiebig, Berger et al. 1990). Despite the fact that MTZ could cure many of the mouse tumour models, clinical trials were terminated when toxicities were seen including prolonged and irreversible bone marrow toxicity, thrombocytopenia which causes bleeding into the tissues, bruising, and slow blood clotting after injury. Following these disappointing results with MTZ, the search for other analogues of MTZ continued looking to retain its good anti-tumour activity but without the toxicity ultimately leading to the development of TMZ (Friedman et al., 2000).

The story of TMZ began in the late 1970s, when Professor Stevens also known as the father of TMZ. Professor Stevens worked at Aston University developing new anti-cancer drugs against cancer. By the late 1980s he and his team developed a promising candidate, which later came to be known as Temozolomide (Sansom, 2009). After a long process of development, TMZ was approved for clinical trials at Charing Cross Hospital in London. Results from these clinical trials with TMZ showed promising results which lead to Cancer Research UK supporting further clinical trials in a greater number of cancer patients. The results showed that the drug could bring significant benefits to patients with brain tumours. As well as extending survival, patients with glioblastoma benefited mentally and physically from treatment with TMZ due to

reduced toxicity. The drug then progressed to the next stage involving much larger phase III trials. After much success, Cancer Research UK helped license the drug leading to the approval of TMZ for use in clinic (Clarke et al., 2010).

In 2001, NICE (the National Institute of Health and Care Excellence) ruled that TMZ could be used for “second line” therapy for people with brain tumours that had come back after treatment. In 2006 the results of the major large scale phase III trial of TMZ alongside radiotherapy for people with glioblastoma brain tumours were announced (Guilfoyle et al., 2011; Mirimanoff et al., 2006). The results found that the drug could bring significant increases in survival in patients with aggressive tumours. As a result of larger trials (Mirimanoff et al., 2006), TMZ was approved by NICE as a front line drug for people newly diagnosed with brain tumours in 2007, and has now been used to treat thousands of patients around the world.

TMZ is a prodrug that undergoes spontaneous decomposition in solution at physiological pH to the reactive intermediate 5-(3-methyl-1-triazenyl)imidazole-4-carboxamide (MTIC), which forms a reactive alkyl diazonium ion that methylates the N^7 and O^6 positions of guanine and the N^3 position of adenine (Tentori and Graziani, 2009). The methyl adducts, O^6-meG, N^7-meG, and N^3-methyladenine (N^3-meA) result in deletion by the mismatch repair (MMR) or O-6-methylguanine-DNA methyltransferase (MGMT) pathways that will lead eventually to cellular apoptosis (Fu et al., 2012) Therefore, TMZ exerts its chemotherapeutic efficacy in cancer cells by inducing cell death. However, acquired resistance to TMZ develops in cancer cells and been shown to participate in the limitation of therapeutic outcome. A large percentage of

tumours are resistant to the cytotoxic effects of the TMZ induced DNA lesion O^6-MeG due to elevated expression of MGMT or a defective MMR pathway.

Whilst being an effective anti-cancer agent, inherent and acquired resistance to TMZ present major obstacles to successful treatment therefore necessitating the need for new and improved analogues that circumvent the drug resistance problem. To understand the mechanisms of resistance in cancer cells the next part of the chapter will focus on the two main types of resistance encountered by TMZ. This predominantly includes MMR and MGMT resistance mechanisms.

MMR resistance in cells

While most DNA replicates without any problems, mistakes do occur, with polymerase enzymes sometimes inserting the wrong nucleotide or too many or too few nucleotides into a sequence (Iyer et al., 2006). Fortunately, most of these mistakes are fixed through various DNA repair processes. DNA mismatch repair (MMR) is one of those systems for recognizing and repairing erroneous insertion, deletion, and miss-incorporation of bases that arise during DNA replication, as well as repairing DNA damage (Pray, 2008).

The MMR system, attempts to repair the mismatch by removing the newly synthesised strand. However if the mismatch remains, DNA damage in the form of single and/or double-strand breaks are generated. The DNA strand breaks are detected by the G2/M check point in the replication cycle and this leads to the induction of apoptosis (Drabløs et al., 2004). Due to the loss of the MMR system the cell cannot detect DNA damage and therefore cannot activate apoptosis leading to drug resistance. The MMR proteins are involved in

mediating the activation of cell cycle checkpoints and apoptosis in response to DNA damage. MMR deficient cells have been reported to be resistant to the methylating agents such as TMZ. The human ovarian adenocarcinoma cell line A2780 is MMR proficient and expresses MLH1. The MLH1 (mutL homologue 1) protein, shown to be important in determining sensitivity to a number of important chemotherapeutic agents. Loss of MMR due to methylation of the hMLH1 gene promoter results in resistance in cancer. Methylation of the hMLH1 gene promoter is observed in many tumour types and is associated with clinical drug resistance (Plumb et al., 2004). A2780/Cis is MMR deficient and does not express MLH1 protein because of hypermethylation of the *hMLH1* gene promoter region (Strathdee et al., 1999).

MGMT resistance in cells

MGMT mediated repair is unique among DNA repair pathways. It transfers the methyl group to an internal cysteine residue in the protein, acting as both a transferor and an acceptor of the alkyl group. MGMT removes the O^6-alkylguanine DNA adduct through covalent transfer of the alkyl group to the conserved active site, cysteine, and restores the guanine to normal (Tiwari and Mishra, 2009). After receiving a methyl group from O^6-meG, MGMT is inactivated and subjected to ubiquitin mediated degradation. Overexpression of MGMT prevents cancer cells from death induced by alkylating agents with a correlation between MGMT activity and tumour drug resistance (Fan et al., 2013). The protective role of MGMT against the cytotoxic effect of methylating drugs such as TMZ has been shown in human cell lines and human xenograft models (Gerson, 2002).

Therefore, investigation of potential drug interactions that modulate MGMT could help improve the efficacy of chemotherapies utilizing alkylating agents (Hegi et al., 2008). Inhibition of MGMT promotes antitumor activity of alkylating agents such as TMZ both *in vitro* and *in vivo* (Dolan et al. 1991; Wedge et al. 1996). Epigenetic silencing of the MGMT gene in tumour cells by methylation of its promoter region has been associated with glioma sensitivity to alkylating chemotherapy in particular TMZ (Esteller et al. 2000). Having identified the mechanism of resistance, MGMT can be successfully inactivated by free guanine base derivatives, with alkyl groups at the O^6 position, which act as "pseudo substrates" (McElhinney et al., 1998). PaTrin2 has been identified as MGMT in-activator and compared to TMZ as a single agent, the combination of PaTrin2 with TMZ has been shown to significantly decrease tumour growth (Middleton et al., 2000). Furthermore, PaTrin2 has entered phase II clinical trials and PaTrin2 plus TMZ combination is under clinical development in England demonstrating the potential therapeutic benefits (Clemons et al., 2005). Cancer Research UK carried out a Phase I clinical study of PaTrin2, which was completed in the early part of 2002. The study showed that the combination of PaTrin2 and Temozolomide was well tolerated. PaTrin2 can effectively inhibit MGMT with no toxicities observed other than those usually seen with TMZ alone (Rietschel et al., 2008).

KuDOS Pharmaceuticals (KuDOS) of Cambridge, United Kingdom acquired a worldwide licence for PaTrin2. KuDOS then went on to pursue Phase II clinical trials with PaTrin2 in combination with Temozolomide against metastatic melanoma and advanced colorectal cancer in 2003 in partnership with Cancer Research UK. To date there have been a number of research groups looking

at PaTrin2 and have published articles in support of the initial result showing that the combination of PaTrin2 and Temozolomide enhancing the antitumor activity overcoming MGMT/MMR resistance (Barvaux et al., 2004b; Clemons et al., 2005; Woolford et al., 2006).

Over the years there has been a focus on utilising PaTrin2 in manipulating MGMT levels to enhance alkylating agent therapy. Research has focused on TMZ, which has presented resistance in many cancer types and therefore the development of PaTrin2 has been valuable. Much of the research carried out with TMZ and PaTrin2 has been in malignant brain tumours (Fan et al., 2013), and much success has been seen against MGMT resistance overcoming significant barriers in treatment (Hegi et al., 2008; Sharma et al., 2009). Current research, phase I and II clinical trials (Kefford et al., 2009; Rietschel et al., 2008) are looking to incorporate PaTrin2 in to standard treatments methods available for the treatment of patients with Gliomas amongst other cancer types.

Current treatment for cancers such as glioblastoma multiforme (GBM), the most common and malignant brain tumour combines chemotherapy involving TMZ, radiotherapy and surgery. TMZ is an alkylating agent that induces apoptosis and is considered to be the frontline chemotherapeutic agent for treatment of GBM (Zhang et al., 2012). Despite TMZ being used as a frontline anti-cancer drug, GBM patients commonly exhibit resistance to TMZ treatment. Therefore a need for better and more improved chemotherapeutic agents may be one of the more productive avenues for improvement. This research is the first to use the novel EA02 drug compounds, which are TMZ derivatives developed in house (by Dr Richard Wheelhouse, School of Pharmacy). These compounds are novel and therefore the mechanism of action is unknown, due to their structural similarities

to TMZ it can proposed that the EA02 drug compounds act in the same way TMZ targets the DNA.

The use of cell lines A2780 and A2780/Cis was important as the mutations possessed by the cancer cells will determine the outcome of the results obtained. The human ovarian adenocarcinoma cell line A2780 is MMR proficient and expresses MLH1, whereas A2780/Cis is MMR deficient and does not express MLH1 protein because of hypermethylation of the *hMLH1* gene promoter region (Strathdee et al., 1999). Furthermore both cell lines A2780 and A2790/Cis express MGMT which contributes towards TMZ resistance (Barvaux et al., 2004b). In terms of properties for any potential analogue of TMZ it would have to possess in the context of MMR/MGMT resistance, the ability to induce lesions which are not recognised by MGMT. The screening process will hope to identify compounds that are not significantly potentiated by PaTrin2 (inactivation of MGMT by PaTrin2 should have little or no effect on activity). In the case of MMR, to identify compound that work or induce toxicity in both MMR proficient and MMR deficient cells. Therefore a compound which is toxic irrespective of cell line and the presence of PaTrin2, will be of interest.

Aims and objectives

In summary, TMZ is a multi-billion dollar drug used to treat glioblastoma. Whilst effective, its efficacy is compromised by drug resistance mechanisms including deficiencies in MMR and over-expression of MGMT. Novel analogues of TMZ will be evaluated with the aim of identifying second generation compounds that retain the desirable properties of TMZ but have improved properties in terms of circumvention of the drug resistance mechanisms that have reduced the efficacy of TMZ. As stated previously, the search for new therapies represents a major strategy towards the treatment of cancer. New chemotherapies can be developed along target orientated principles or by developing analogues of successful drugs that have been engineered to provide solutions to problems encountered by the parent drug, Temozolomide.

The specific aims of the work conducted in this chapter are listed below:

4. To determine the activity of TMZ, MTZ, Cisplatin and Melphalan in the absence and presence of PaTrin2 against a panel of cell lines *in vitro*.
5. To determine whether the activity of novel EA02 drug compounds can overcome MMR and MGMT resistance against a panel of cell lines *in vitro*.
6. To determine whether inhibition by PaTrin2 increases sensitivity to TMZ, MTZ, Cisplatin, Melphalan and the novel EA02 drug compounds *in vitro*.

Methods and Materials

Consumables

All chemicals and drugs were purchased from Sigma Aldrich (Poole, United Kingdom) unless stated otherwise. All cell culture consumables were purchased from Corning (Amsterdam, The Netherlands). The EA02 compounds and PaTrin2 were provided by Dr Richard Wheelhouse (University of Bradford, UK)

Cell Lines and Maintenance

The A2780 and the Cisplatin resistant A2780/Cis human ovarian cancer cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC). All cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium, supplemented with 10% foetal bovine serum, sodium pyruvate (1mM) and L- Glutamine (2mM). Cells were maintained at 37˚C in a 5% CO_2 humidified atmosphere until required for experimental use. For routine sub-culturing, cell counting, cell viability assays and the MTT assay, please refer to the methods sections in Chapter two.

Measuring the response of cell lines to Temozolomide, Mitozolomide, Cisplatin and a range of EA02 compounds

The response of A2780 and A2780/Cis following continuous exposure to a broad range of concentrations of Temozolomide (TMZ), Mitozolomide (MTZ), Melphalan and Cisplatin were determined using the MTT assay as described previously in Chapter 2. Similarly, the response of A2780 and A2780/Cis

following continuous exposure to EA02 drug compounds was determined using the MTT assay. A2780 and A2780/Cis cell lines were exposed to a broad range of drug concentrations for 6 days, details of which are presented in the results section. All compounds were initially dissolved in DMSO and the final concentration of DMSO was 0.1% (v/v) in all cases. As described in chapter 2, the results are expressed in terms of IC_{50} values and each experiment was repeated in triplicate.

Combination studies to determine the effect of PaTrin2 on the activity of TMZ, MTZ, Melphalan, Cisplatin and EA02 compounds

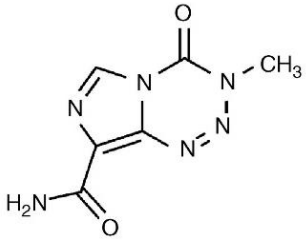
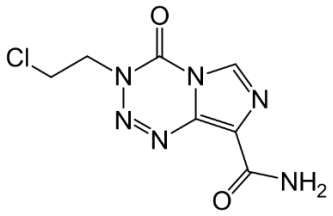
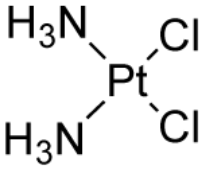
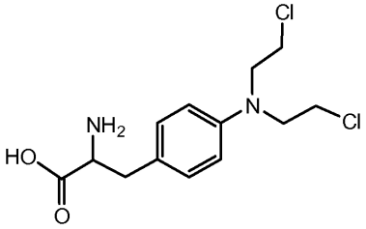
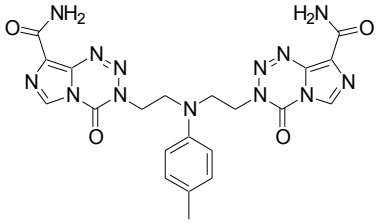
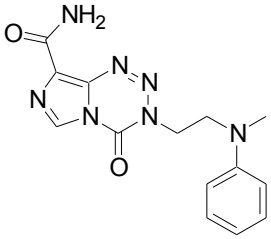
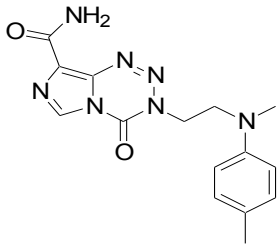
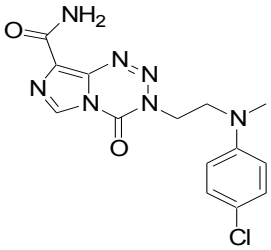
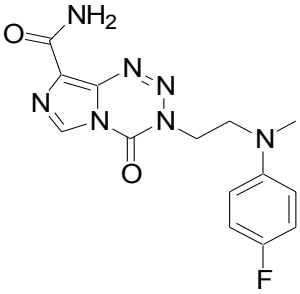
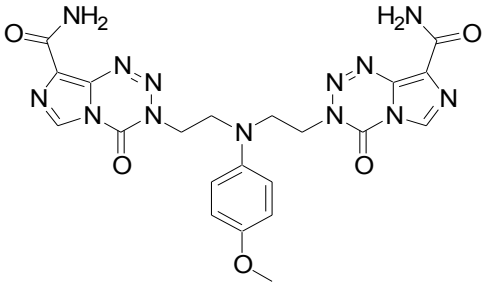
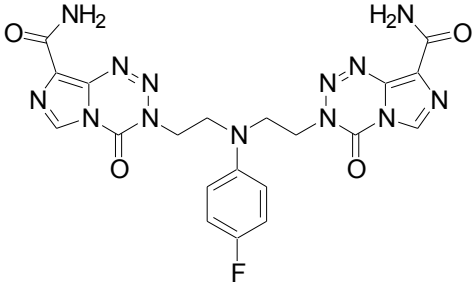
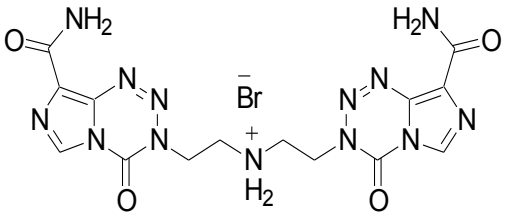
The response of A2780 and A2780/Cis following continuous exposure to TMZ, MTZ, Cisplatin, Melphalan and EA02 drug compounds in combination with PaTrin2 was determined using the MTT assay. Details of the compounds used and their solubility is presented in table 4. A diluent was made consisting of complete RPMI 1640 culture medium plus PaTrin2 (10μM in DMSO). This diluent was used to make up the highest concentration of the compounds being tested and also used for making up the remaining 8 concentrations using a 2 fold dilution. For combination studies there were variations from the standard chemosensitivity assays described previously. The first variation was the inclusion of an additional control. In addition to the routine drug free control, an additional control was included which consisted of cells exposed to PaTrin2 only. For the calculation of % cell survival, this second control was used to represent 100% cell survival (ie response was normalised relative to the effect of PaTrin2 alone). All cells were exposed to compounds (with or without PaTrin2) for 144

hours and response was determined using the MTT assay as described previously. Any deviation in the dose response curve from the drug alone experiments (i.e. no PaTrin2) indicates either antagonism or synergistic effects.

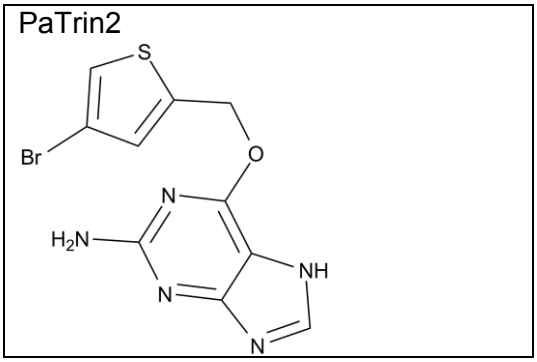
Table 4: Compounds used for testing the effect of PaTrin2 on chemosensitivity.

DRUG	MOLECULAR WEIGHT	SOLVENT USED TO SOLUBILIZE COMPOUNDS	STOCK CONCENTRATION	STORAGE
Temozolomide	194.15	DMSO	100mM	-20ºC
Mitozolomide	242.62	DMSO	100mM	-20ºC
Cisplatin	300.05	DMSO	100mM	-20ºC
Melphalan	305.20	WATER	100mM	4ºC
EA02-45	527.70	DMSO	10mM	-20ºC
EA02-58	314.62	DMSO	100mM	-20ºC
EA02-59	348.21	DMSO	100mM	-20ºC
EA02-60	357.97	DMSO	100mM	-20ºC
EA02-61	345.97	DMSO	100mM	-20ºC
EA02-64	540.44	DMSO	20mM	-20ºC
EA02-65	534.22	DMSO	20mM	-20ºC
EA02-27C	628.54	DMSO	20mM	-20ºC
PaTrin-2	333.37	DMSO	10mM	-20ºC

*For information on the characterisation of the above compound please refer to the following article (Pletsas et al., 2013)

Temozolomide 	Mitozolomide 
Cisplatin 	Melphalan 
EA02-45 	EA02-58 
EA02-59 	EA02-60 
EA02-61 	EA02-64 
EA02-65 	EA02-27c 

PaTrin2



Results

The response of A2780 and A2780/Cis cells following continuous exposure to MTZ, TMZ, Cisplatin, and Melphalan in the absence and presence of PaTrin2 are presented in figure 3.1 and table 5. Both A2780 and A2780/Cis cell lines were sensitive to all the drugs with a broad range of IC_{50} values obtained. TMZ did not achieve an IC_{50} when exposed to A2780 cells in the absence of PaTrin2 but in the presence of PaTrin2, TMZ showed toxicity against the cell line A2780 (IC50 = 17.22uM). The response of A2780 and A2780/Cis cells following continuous exposure to MTZ, TMZ, Cisplatin, and Melphalan in the presence of PaTrin2 generated a broad range of IC_{50} values. Some but not all the drugs were potentiated and for those of the drugs which were potentiated, a leftwards shift was observed in the dose response curve.

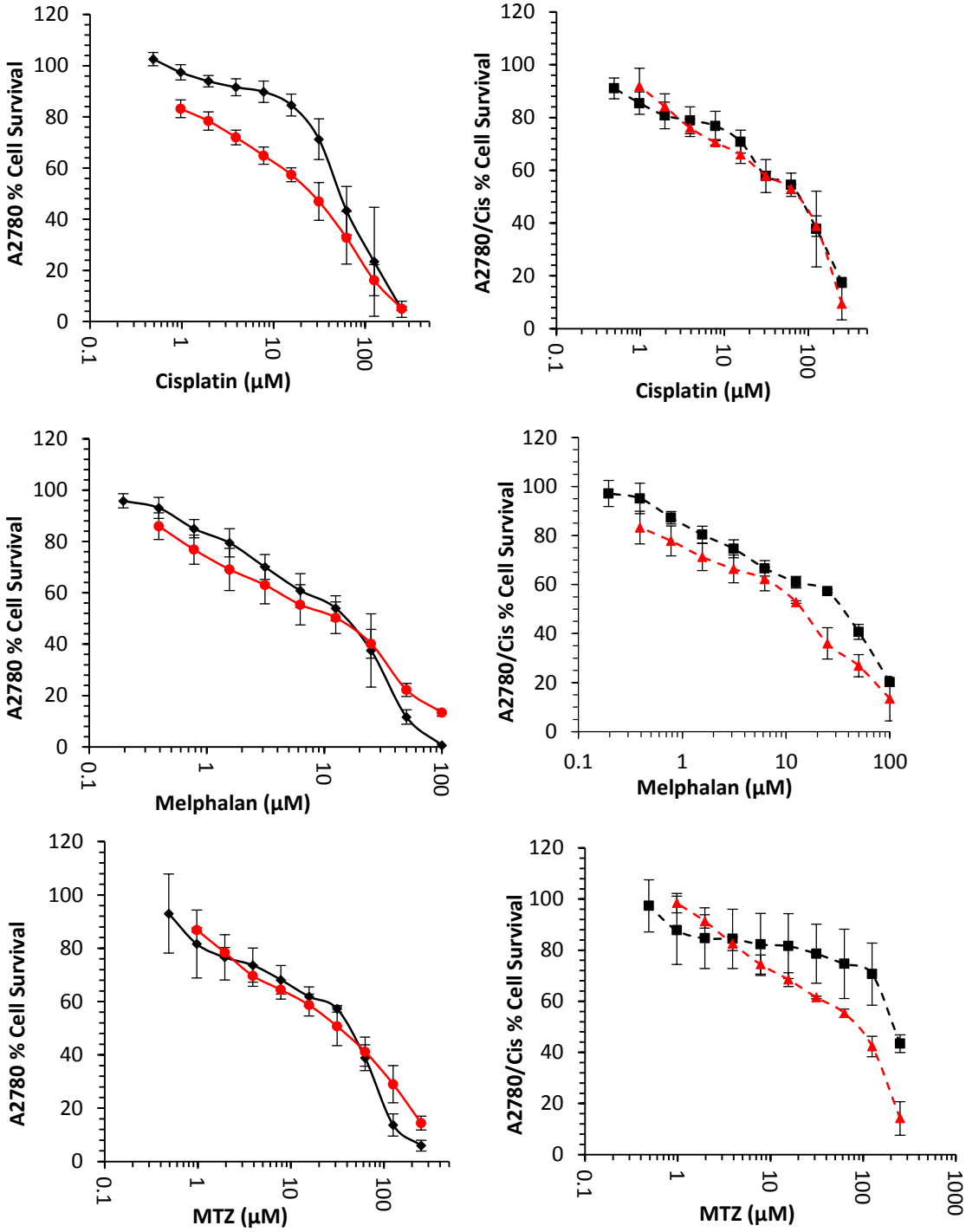


Figure 3.1: Response of A2780 and A2780/Cis cell lines to TMZ, MTZ, Melphalan and Cisplatin alone (solid black line-A2780, solid red line-A2780 in the presence of PaTrin2) and in combination with Patrin2 (dotted black line-A2780/CIS, dotted red-A2780/CIS in the presence of PaTrin2). Each value represents a mean ± SD for three independent experiments.

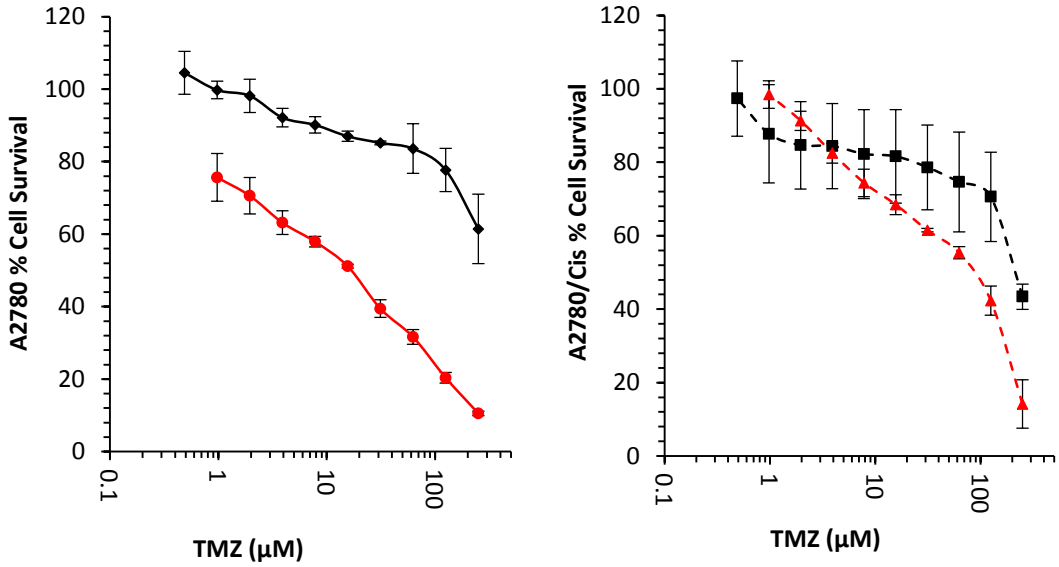


Figure 3.1: (Continued) Response of A2780 and A2780/Cis cell lines to TMZ, MTZ, Melphalan and Cisplatin alone and in combination with PaTrin2.

Table 5: Summary of IC_{50} values for drug alone and drug in combination with PaTrin2

Drug	A2780 IC_{50} (drug only) µM	A2780 IC_{50} (drug + PaTrin2) µM	A2780 Cis IC_{50} (drug only) µM	A2780 Cis IC_{50} (drug + PaTrin2) µM
Cisplatin	23.87±03.88	11.38±03.34	40.95±03.28	32.24±06.13
Melphalan	16.84±01.47	14.50±05.59	36.05±06.08	15.99±03.07
Mitozolomide	44.28±03.88	28.73±10.79	72.70±01.73	30.42±05.47
Temozolomide	>250	17.22±0.95	216.87±14.34	88.90±03.29

Response of A2780 and A2780/Cis to EA02 compounds

The response of A2780 and A2780/Cis cell lines following continuous exposure to EA02 drug compounds in the absence and presence of PaTrin2 are presented in figures 3.2, figure 3.3 and table 6. In order of potency in response to A2780 cell line, the drug compound EA02-64 was the most potent followed by EA02-65, 45 and 27c with IC_{50} values of 2.66±2.85μM, 10.94±3.19μM, 11.27±2.40μM and 14.90±1.53μM respectively. The response to A2780/Cis cell line in order of potency were as follows: 3.06±1.93μM (EA02-65), 9.77±3.38μM (EA02-64), 22.97±6.08μM (EA02-45) and EA02-27c no IC50 was obtained (>40μM). Over all A2780/Cis was more resistant to the EA02 drug compounds than A2780 cells (table 6). A2780 and A2780/Cis cell lines were sensitive to all the drug EA02 drug compounds plus PaTrin2 with varying degrees of toxicity. In the presence of PaTrin2 cell lines A2780 and A2780/Cis showed greater sensitivity in response to EA02-45, 64, 65 and 27c with the exception of EA02-64, which did not achieve an IC_{50} value in response to A2780/Cis cell line. In the presence of PaTrin2 cell line A2780 showed greater sensitivity.

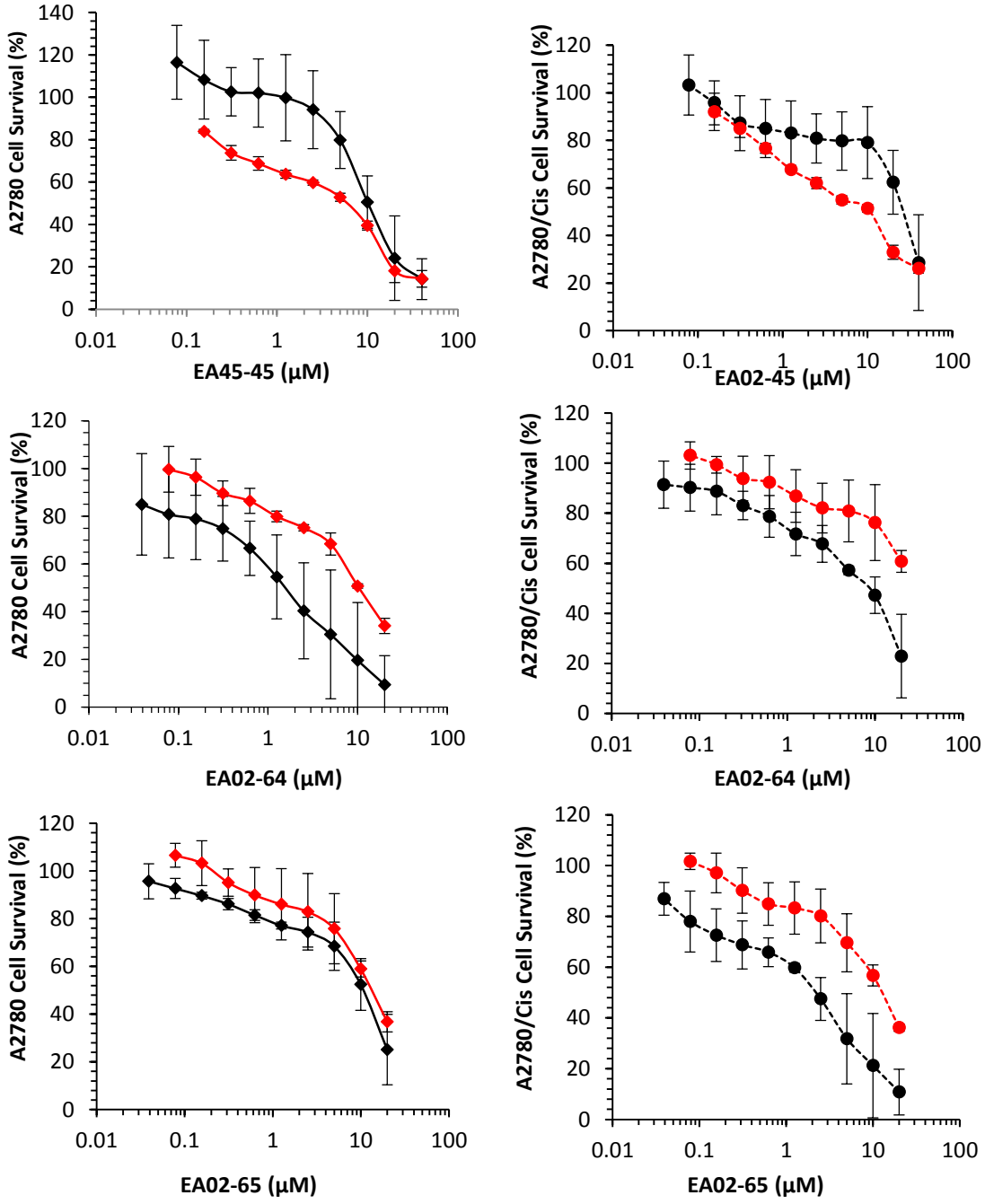


Figure 3.2: The response of A2780 and A2780/Cis cells following continuous exposure to EA02 compounds with or without PaTrin2. The black and red symbols and lines represent EA02 compounds only and EA02 plus PaTrin2 respectively. The label on the Y axis denotes the cell line used and the X axis details the specific EA02 compound tested. Each value represents the mean ± standard deviation for three independent experiments.

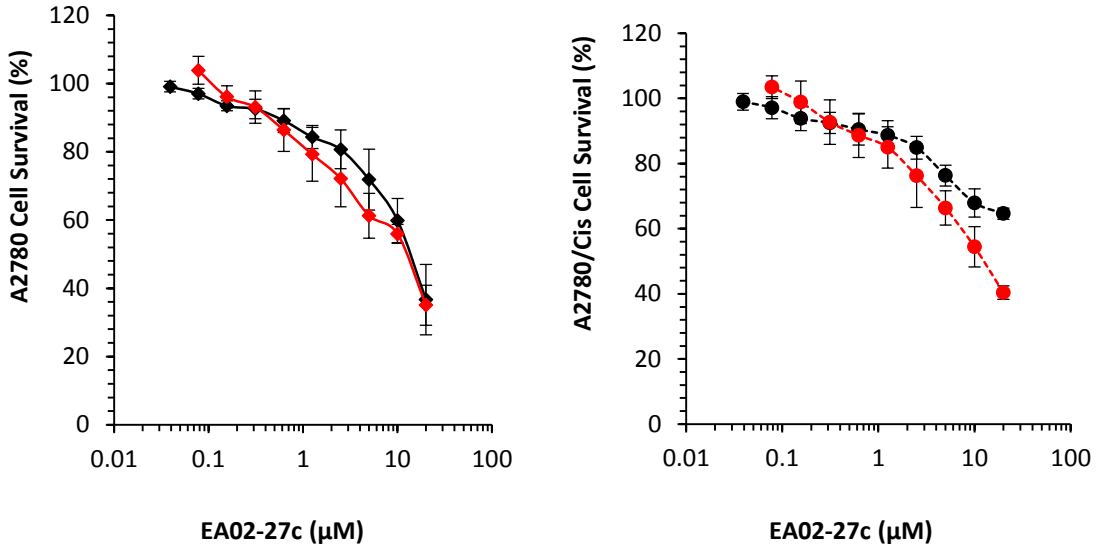


Figure 3.2: (Continued) The response of A2780 and A2780/Cis cells following continuous exposure to EA02 compounds with or without PaTrin2.

Table 6: Summary of IC_{50} values for drug alone and drug in combination with PaTrin2

Drug	A2780 IC_{50} (drug only) µM	A2780 IC_{50} (drug + PaTrin2) µM	A2780 Cis IC_{50} (drug only) µM	A2780 Cis IC_{50} (drug + PaTrin2) µM
EA02-27c	14.90±01.53	14.06±01.24	>40	12.28±02.66
EA02-45	11.27±02.40	06.02±00.65	22.97±06.08	10.76±00.59
EA02-64	02.66±02.85	10.53±00.64	09.77±03.38	>40
EA02-65	10.94±03.19	12.94±01.94	03.06±01.93	13.10±01.72

The response of A2780 and A2780/Cis cells following continuous exposure to EA02 drug compounds EA02- 58, 59, 60, 61 and 66 is presented in figure 3.3. Both A2780 and A2780/Cis cells were sensitive to all the drugs with varying degrees of sensitivity. In the order of potency, EA02-59, 60, 66, 58 and 61 were the most potent against A2780 cells. Against A2780/Cis cells, EA02-61, 59, 60, 58 and 66 were most potent. Both cell lines responded differently in response to EA02 drug compounds, A2780/Cis cells were more resistant compared to A2780 cells as IC_{50} values in response to A2780/Cis were higher in comparison to IC_{50} values in response to A2780 except EA02-61 (see table 7). None of the EA02 drugs mentioned above worked well against both cell lines.

In the presence of PaTrin2 in order of potency EA02-66, 58, 59, 60 and 61 were potent in response to A2780 cells and in order of potency EA02-66, 60, 59 and 58 were the most potent with the exception of EA02-61 where no IC_{50} values was obtained. A2780/Cis cells were more resistant than A2780 once again. In the presence of PaTrin2 IC_{50} values in response to A2780/Cis were higher in comparison to IC_{50} values in response to A2780 except EA02-61 (see table 7). PaTrin2 altered the response of cells to some but not all individual EA02 compounds, in response to A2780 the following were potentiated by PaTrin2: EA02-58, 61 and 66 and in response to A2780/Cis the following were potentiated by PaTrin2: EA02-66, the remaining EA02 drug compounds produced higher IC_{50} values in the presence of PaTrin2 in comparison to EA02 drug alone.

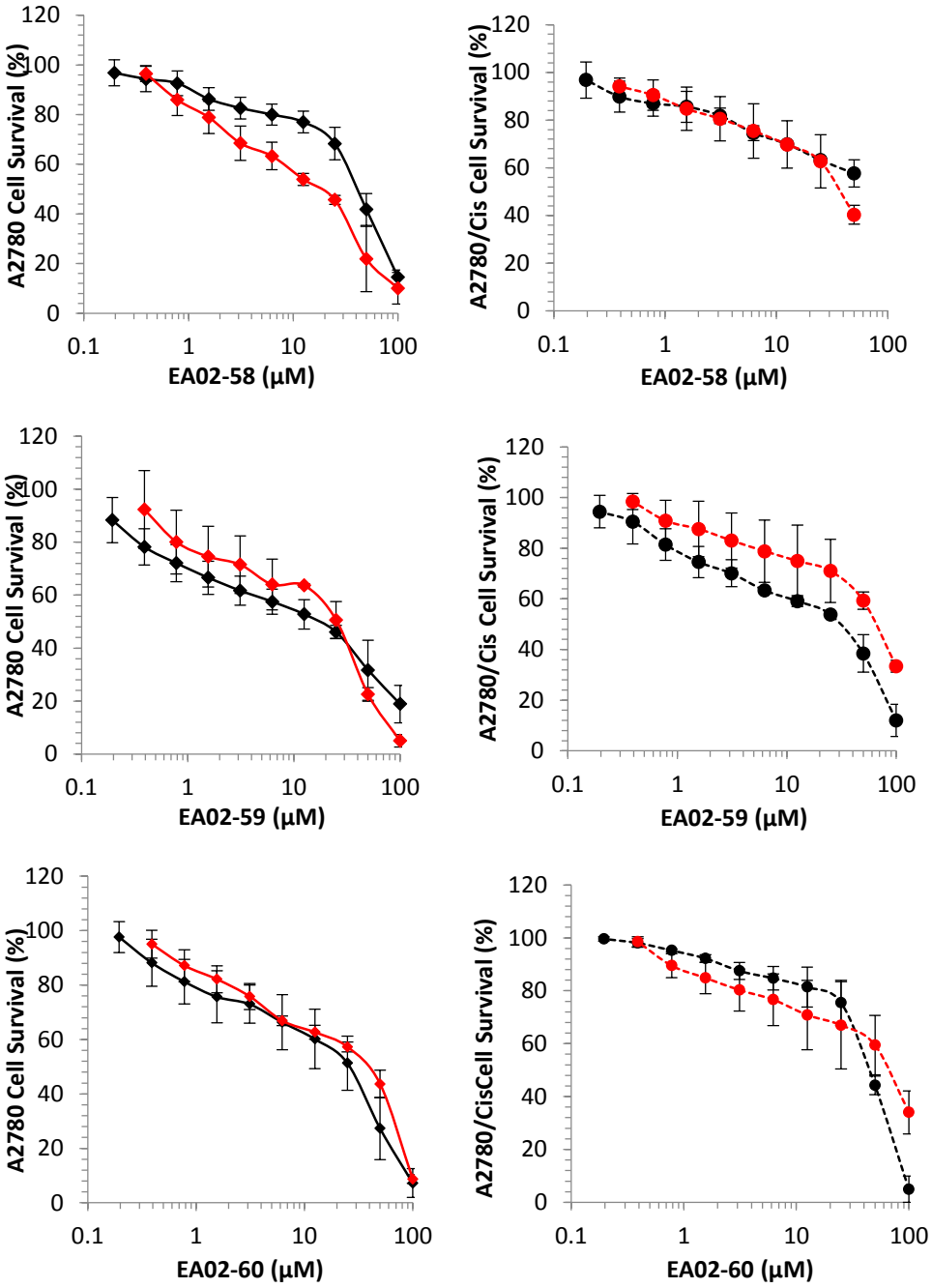


Figure 3.3: The response of A2780 and A2780/Cis cells following continuous exposure to EA02 compounds with or without PaTrin2. The black and red symbols and lines represent EA02 compounds only and EA02 plus PaTrin2 respectively. The label on the Y axis denotes the cell line used and the X axis details the specific EA02 compound tested. Each value represents the mean ± standard deviation for three independent experiments.

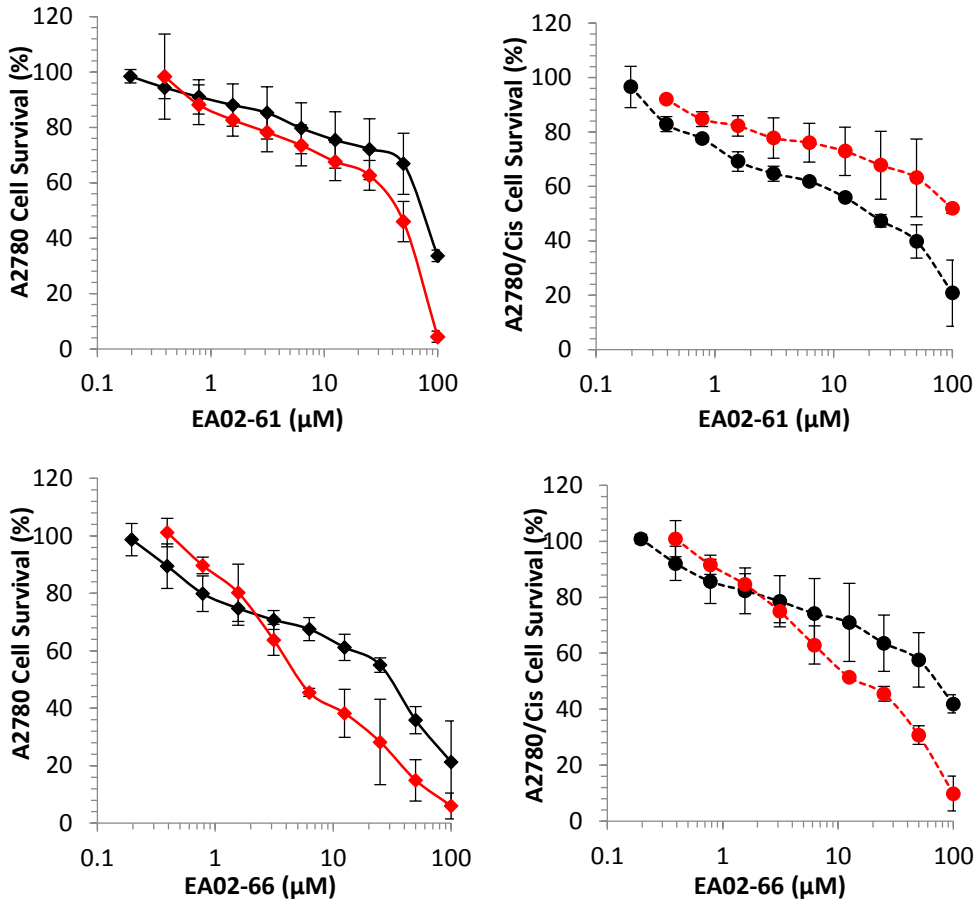


Figure 3.3: (continued) The response of A2780 and A2780/Cis cells following continuous exposure to EA02 compounds with or without PaTrin2.

Table 7: Summary of IC_{50} values for drug alone and drug in combination with PaTrin2

Drug	A2780 IC_{50} (drug only) µM	A2780 IC_{50} (drug + PaTrin2) µM	A2780 Cis IC_{50} (drug only) µM	A2780 Cis IC_{50} (drug + PaTrin2) µM
EA02-58	42.37±06.35	18.37±02.00	64.41±02.49	74.96±13.42
EA02-59	15.81±04.49	25.31±04.62	32.47±05.06	64.76±04.12
EA02-60	29.46±06.03	40.21±06.41	45.68±01.90	64.76±07.83
EA02-61	73.25±10.22	43.98±09.64	21.54±02.80	>100
EA02-66	31.63±02.76	5.46±00.08	72.69±12.04	15±03.29

Discussion

The objectives set out for this chapter were to identify novel analogues of TMZ that circumvent the resistance mechanisms that limit the effectiveness of TMZ. As outlined in the introduction, the ideal compound would be one that induces cytotoxicity that is not MGMT dependent. In the screen, this would be identified as compounds that induces similar IC_{50} values in the presence and absence of the MGMT inhibitor PaTrin2. In addition, compounds that also work against MMR deficient cells (A2780/Cis) would be desirable. The results obtained in this chapter are discussed in the context of these requirements.

The response from A2780 and A2780/Cis cells following continuous exposure to the drugs was measured via MTT assay. TMZ was non-toxic against A2780 cells yet toxic against A2780/Cis cells, whereas MTZ, Cisplatin and Melphalan were all toxic to both cell lines A2780 and A2780/Cis. In the absence of PaTrin2, cells from cell line A2780/Cis demonstrated greater resistant to MTZ, Cisplatin and Melphalan in comparison to cell line A2780 (table 3.3). In the presence of PaTrin2 TMZ, MTZ, Cisplatin and Melphalan were all toxic and displayed potentiation when exposed to A2780 and A2780/Cis cell lines (please refer to table 5) demonstrating that the addition of PaTrin2 enhanced cytotoxic activity indicating a role for MGMT in the mechanism of action. In the absence of PaTrin2, TMZ did not induce toxicity but in the presence of PaTrin2, TMZ was able to achieve an IC_{50} value. This result is consistent with a role for MGMT in conveying drug resistance as has been described elsewhere in the literature (Hegi et al., 2008; Zhang et al., 2011). PaTrin2 has been found to potentiate the activity of certain drugs, found in the study conducted by Baravaux et.al.

(Barvaux et al., 2004a) reinforcing that it is a valuable drug in the fight against resistance and a drug with great therapeutic potential. In summary, inclusion of PaTrin2 in cell culture media potentiated the activity of MTZ, TMZ, Cisplatin and Melphalan with the most marked effect being observed in the case of TMZ. These results also provided evidence to validate the experimental conditions required to critically assess the activity of novel analogues.

Compared to the A2780/Cis cell line, A2780 cells were more sensitive. IC_{50} values obtained were higher in response to A2780/Cis compared to IC_{50} values obtained against cell line A2780.

Some but not all EA02 drug compounds were of interest, as stated previously in the introduction in terms of properties for any potential analogue of TMZ it would have to possess in the context of MMR/MGMT resistance, the ability to induce lesions which are not recognised by MGMT. In the case of MMR, to identify compound that work or induce toxicity in both MMR proficient and MMR deficient cells. The following compounds were toxic in the absence of PaTrin2; EA02-45, 59, 60, 64, 65 and 27c against cells from cell line A2780 (table 6 and 7). EA02-45, 59, 61, 64 and 65 were toxic in the absence of PaTrin2 against cells from cell line A2780/Cis (table 6 and 7). From the range of EA02 drugs mentioned above, only a few of the drugs were effective in terms of overcoming MGMT resistance or did not require a MGMT inhibitor (PaTrin2). In terms of MMR resistance and looking for compounds which induced toxicity irrespective of deficient or proficient MMR, the following EA02 compounds were promising; EA02-45, 59, 64 and 65 producing toxicity irrespective of MGMT or MMR status. Therefore the aims of generating new TMZ like (imidazotetrazine) prodrugs with

activity independent of MGMT and MMR have been achieved via selected EA02 drug compounds.

Although we look to find and identify compounds which are not dependent on PaTrin2 current literature states that there is a use for PaTrin2. The synergistic effects of PaTrin2 are well documented in combination with TMZ, current research is looking to combine PaTrin2 with TMZ to achieve the optimum toxicity and where appropriate in conjunction with radiotherapy (Kefford et al., 2009).

Future research

The initial success of identifying and the development of EA02 drug compounds which have the desired characteristics in terms of overcoming MGMT/MMR resistance in the form of EA02-45, 59, 64 and 65 provides scope for testing on different cancer cell types/tumour types and observe how tumour respond to such drugs. Furthermore the EA02 compounds can be tested against the HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines to see whether the p53 status of cell influences the cytotoxic nature of these drugs.

CHAPTER FOUR

Discussion

Increasing evidence suggests that oncoproteins can directly reprogram tumour cell metabolism, rendering the cells addicted to certain nutrients in a way non-transformed cells are not. However, whether alterations in cancer metabolism can be safely targeted therapeutically remains be determined. In chapter 2 of this thesis, the topic of interest focused on whether targeting key enzymes involved in the Warburg effect could circumvent resistance to apoptosis (conferred by cells switching to a glycolytic phenotype) and potentiate the activity of existing anti-cancer drugs. In addition, tumour cells become 'addicted' to glutamine as part of the Warburg effect and glutamine depletion studies were conducted to determine whether or not this strategy was a feasible option for therapeutic intervention. Finally, chapter 3 of this thesis addressed another major theme which was the evaluation of novel analogues of TMZ to identify analogues, which evade the key mechanisms that cause resistance to TMZ. Throughout this discussion, these three themes will be discussed and future work proposed to progress each topic individually or to forge links between the themes.

Manipulation of the Warburg effect to enhance the activity of anti-cancer drugs

As described in chapters 1 and 2, one of the advantages cancer cells gain by switching to aerobic glycolysis is avoidance of apoptosis by altering mitochondrial membrane physiology. This not only provides cells with a selection advantage favouring cell survival but it also confers resistance to

therapeutic drugs. The hypothesis being tested in this and other studies is whether or not manipulation of the Warburg effect modifies mitochondrial function leading to reactivation of apoptosis (Michelakis et al., 2008). Inhibition of LDH-A and/or PDK1 have been shown to promote the activity of certain cytotoxic drugs by reactivating apoptosis and this is confirmed in the case for Etoposide (figure 2.11). Using DCA to inhibit PDK1 (figure 2.10), increased apoptosis was seen following the treatment of cells with Etoposide compared to treatment of cells with Etoposide alone (figure 2.11). Similar results have been obtained for a range of cytotoxic drugs (Heshe et al., 2011; Michelakis et al., 2010) but no studies to my knowledge have looked at the effect of inhibiting LDH-A and/or PDK1 on the activity of targeted therapeutics. In this study, combination of Bortezomib, Gefitinib, Imatinib, Masitinib, Nilotinib and Sorafenib, with DCA against the HCT116 $p53^{+/+}$ and the combination of Bortezomib, Dasatinib, Gefitinib, Imatinib, Masitinib, Nilotinib, Sunitinib, and Vandetanib with DCA significantly potentiated the activity of DCA (figure 2.8 and table 3). It is possible that a similar mechanism to that proposed for cytotoxic drugs (i.e. reactivation of apoptosis following manipulation of the Warburg effect) is responsible and analysis of apoptosis induction is required as future work. It is also conceivable that alternative explanations exist. For example, it is not known what happens to the target specific signalling pathways following inhibition of LDH-A and/or PDK1 and further studies are required to explore these mechanisms. In summary, the results of this study have demonstrated for the first time that targeted therapeutic drugs have an effect on DVA activity and further studies are required to explore this further. This includes understanding the mechanistic basis responsible for the observed effects and investigation of other pharmacological variables such as scheduling (i.e. do you see the

enhanced effects only after inhibition of the Warburg effect) and keeping DCA at a constant concentration whilst varying TKI concentrations. Further studies to evaluate effects on other targeted therapeutics are also required in a range of cell types with different oncogenic drivers of the Warburg effect.

Manipulating the levels of glutamine to determine how much glutamine needs to be depleted in order to reduce the growth or kill tumour cells

As indicated in chapter 1 and summarised in figure 2.1, there are two major molecules that are catabolized in substantial quantities in cancer cells, namely glucose and glutamine (Wise and Thompson, 2010). Glucose and glutamine supply the majority of the carbon, nitrogen, free energy and reducing equivalents necessary to support cell growth and division in proliferating cells (Dang et al., 2009). Research in to glutamine has provided evidence that glutamine has become “conditionally essential” during inflammatory conditions such as infection and injury (Newsholme, 2001) under healthy development. It is well documented that under appropriate conditions, glutamine is essential for cell proliferation, and that it can enhance the function of stimulated immune cells. On the other hand glutamine is essential for the proliferation of most cells and the viability of some cancer cells (Daye and Wellen, 2012). Glutamine metabolism contributes to the ability of cancer cells to continuously grow and proliferate by supporting ATP production and biosynthesis of proteins, lipids, and nucleic acids (DeBerardinis and Cheng, 2010) and also modulates redox homeostasis and can impact the activity of signal transduction pathways (Frezza and Gottlieb, 2009). Therefore understanding and learning about the behaviour of cancer cells in response to glutamine depletion remained important. The

experimental data obtained from the panel of cancer cell lines suggests that cancer cells require glutamine or are dependent on glutamine (figure 2.13). One strategy for targeting this property is to deplete glutamine and restrict tumour cell growth by eliminating this essential nutrient. The data presented in chapter 2 demonstrates that glutamine depletion does reduce the growth of tumour cells in a dose dependent manner in the majority of cell lines except for A549 (figure 2.13 and 2.14). Whilst the growth of A549 cells was reduced in glutamine-deprived media, they still remained viable and capable of some growth. For the majority of cell lines tested however, glutamine deprived media had a significant effect on cell growth and caused cell death in many cells. In order to induce cell death however, glutamine levels had to be severely depleted and maintained at this level for a substantial time (figure 2.14). If glutamine was re-introduced to cells following a period of deprivation, cells were able to recover and proliferate (figure 2.15). These results raise two important questions with regards to glutamine deprivation as a strategy for treating cancer. First, can glutamine levels be reduced to the very low levels required to induce cell kill and second, can low levels of glutamine be maintained at such low levels for long enough to have a cytotoxic effect?

The data presented in this thesis suggest that achieving these low levels of glutamine for prolonged periods of time would be challenging suggesting that glutamine deprivation alone is unlikely to be effective. In combination with other strategies targeting glutamine biochemistry (eg inhibitors of glutamine transport or glutamine metabolism), glutamine deprivation may enhance activity and further studies to explore this proposal are required. In summary, glutamine deprivation alone is unlikely to be effective as it is doubtful that complete

deprivation can be achieved *in vivo* and maintained for long enough to have a cytotoxic effect.

Evaluating potential analogues of TMZ in the context of MMR/MGMT resistance

TMZ is a prodrug that undergoes spontaneous decomposition to the reactive intermediate MTIC, which methylates the N^7 and O^6 positions of guanine and the N^3 position of adenine (Tentori and Graziani, 2009) causing methylation of the DNA and ultimately apoptosis. However, acquired resistance to TMZ has developed in many cancer cells and been shown to participate in the limitation of therapeutic outcome. Whilst being an effective anti-cancer agent, inherent and acquired resistance to TMZ present major obstacles to successful treatment therefore necessitating the need for new and improved analogues that circumvent the drug resistance problem. Identifying novel analogues of TMZ that retain the desirable properties of TMZ but have improved properties in terms of circumvention of drug resistance mechanisms was the aim.

The novel analogues of TMZ were synthesised by Dr Richard Wheelhouse in Bradford and these were evaluated against two cell lines. A2780 is proficient in mismatch repair (MMR) whereas the A2780/Cis cell line is deficient in MMR. The other resistance mechanism for TMZ is MGMT and both A2780 and A2780/Cis are MGMT proficient. The MGMT inhibitor PaTrin2 was used to determine whether or not the activity of compounds is dependent on MGMT. Before setting out on the screening of compounds, the desired selection criteria were activity against both A2780 and A2780/Cis cells (demonstrating that the compounds activity was independent of MMR status) and no effect on cellular

response when compounds were incubated in the presence of PaTrin2 (demonstrating that the compounds are MGMT independent). The results of this study demonstrated that EA02-45, 59, 64 and 65 induced cellular toxicity profiles that were independent of the MMR and MGMT status. The exact mechanisms by which these compounds evade these resistance mechanisms remains to be determined and further evaluation of these compounds is required. As TMZ has very good pharmacological properties *in vivo*, additional studies are required to evaluate the properties of the derivatives. In addition, recent studies have demonstrated that TMZ promotes genomic instability in patients with glioblastoma (Laigle-Donadey et al., 2010) leading to the emergence of a more aggressive disease. Additional studies are required to determine whether or not these new derivatives of TMZ cause similar effects or are less mutagenic than TMZ. In conclusion, the results of this thesis have identified novel analogues of TMZ that could have better properties in terms of circumvention of drug resistance mechanisms. This represents the start of a journey towards the development of second generation TMZ drugs that could have therapeutic value.

Finally, there is scope to merge the two major themes explored in this thesis. As manipulation of the Warburg effect can affect key processes such as apoptosis leading to the enhancement of anti-cancer activity *in vitro*, combinations of inhibitors of LDH-A/PDK1 with TMZ and its derivatives would be an additional area for future work. Because of time constraints in this thesis, such studies were not conducted here but are planned for the future. What this thesis has demonstrated however is the potential to use the cancer cells biochemical

properties to enhance the activity of anti-cancer drugs and this is the major conclusion arising from this work. There are many challenges ahead in terms of translating this into *in vivo* studies but the results of this thesis suggest that such studies are warranted as the next step in the process of ultimately developing better therapeutic strategies for treating patients.

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